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MICROCONTAINERS FOR ORAL VACCINE DELIVERY

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PhD thesis

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IDUN

VILLUM FONDEN

"In God we trust, all others must bring data."

W. Edwards Deming

MICROCONTAINERS FOR ORAL VACCINE DELIVERY

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PREFACE

The scientific work presented in this thesis was carried out in the Nanoprobes group at the Department of Micro- and Nanotechnology, Technical University of Denmark, Copenhagen, Denmark, and at New Zealand's National School of Pharmacy, University of Otago, Dunedin, New Zealand. Two stays of 4 and 9 months, respectively were spend in Professor Sarah Hooks group at University of Otago in New Zealand.

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ABSTRACT

The positive impact of vaccines on global human health is second only to clean drinking water, sanitation and hygiene. The emphasis on safety in modern vaccines has motivated use of highly purified antigens to create subunit vaccines. However, these need to be formulated with adjuvants to be effective. Most vaccines require cold chain distribution and are administered via injection by trained healthcare personnel. This is logistically and economically challenging causing many people to be insufficiently vaccinated. Temperature stable oral subunit vaccines are ideal to overcome these challenges, but subunit vaccines require sophisticated oral delivery systems to survive the harsh conditions of the gastrointestinal tract. Vaccines in powder form are most suitable for use with such delivery systems and are often more stable than liquid forms.

In this PhD project, a subunit vaccine formulation was designed using the protein ovalbumin as model antigen and an adjuvant system composed of cubosomes with Quil-A. Spray drying was used to produce a powder that formed the particulate vaccine formulation upon hydration. The vaccine was evaluated *in vitro* and showed good properties for vaccination. In addition, the antigen was stable in the powder during dry storage at 25°C for at least 6 months. A design of experiments approach was used to investigate the effects of four important spray drying parameters on key product characteristics. Input-output correlations were established and it was concluded that the method is robust with little impact of the parameters on vaccine-related characteristics thus allowing optimisation to focus on process or powder characteristics. The vaccine was highly immunogenic *in vivo* in mice when administered by s.c. injection, but ineffective following oral administration.

The main goal of this PhD project was to evaluate microcontainers as oral vaccine delivery system. Microcontainers are small cylindrical polymer-reservoirs with an opening at one end. These were filled with the vaccine and then sealed with a pH-sensitive lid to carry the vaccine safely through the stomach and release it in the intestine. *In vitro* studies indicated that the microcontainers could protect the vaccine from the challenges of the gastrointestinal tract and deliver it safely to the small intestinal wall. However, they were unable to improve the oral immunogenicity of cubosomes with OVA and Quil-A *in vivo*. These results indicate that oral delivery systems such as microcontainers should be used to make vaccines with weak oral immunogenicity more potent rather than to deliver orally ineffective vaccines.

DANSK RESUME

Vacciner har haft stor positiv indflydelse på den globale menneskelige sundhed kun overgået af rent drikkevand, kloakering og hygiejne. Den store fokus på sikkerhed i moderne vacciner har motiveret brug af oprensede antigener til at skabe subunit vacciner. Disse skal dog formuleres med adjuvanter for at være effektive. De fleste vacciner skal distribueres på køl og indgives ved indsprøjtning af trænet sundhedspersonale. Dette giver anledning til logistiske og økonomiske udfordringer som gør at mange mennesker forbliver utilstrækkeligt vaccineret. Orale vacciner der er stabile over for temperaturudsving er ideelle til at omgå disse udfordringer, men subunit vacciner kræver avancerede leveringssystemer for at kunne overleve de hårde betingelser i mavetarm kanalen. Vacciner på pulver form er velegnede til brug med sådanne systemer og er ofte mere stabile end vacciner på flydende form.

I dette Ph.d. projekt blev en subunit vaccineformulering designet med proteinet ovalbumin som model antigen og et adjuvant system bestående af cubosomes med Quil-A. Spray tørring blev benyttet til at producere et pulver, der dannede den partikulære vaccine formulering ved hydrering. *In vitro* viste vaccinen gode egenskaber for vaccinering og antigenet var stabilt i pulveret i mindst 6 måneder ved tør opbevaring ved stuetemperatur. En design of experiments tilgang til forsøgsplanlægning blev benyttet til at undersøge effekten af fire spraytørringsparametre på vigtige produkt egenskaber. Input-output korrelationer blev etableret og det kunne generelt konkluderes, at metoden var robust, med lille indvirkning af parametrene på vaccine relaterede produkt egenskaber. Ved optimering kan der derfor fokuseres på proces- og pulver egenskaber. Vaccinen var effektiv *in vivo* i mus efter subkutan administrering, men havde ingen effekt oralt.

Hovedformålet med denne Ph.d. var at evaluere mikrocontainere til oral vaccinelevering. Mikrocontainere er små cylindriske polymer beholdere med en åbning i den ene ende. Disse blev fyldt med vaccine og lukket med et pH-følsomt låg for at bringe vaccinen sikkert igennem maven og frigive den i tarmen. *In vitro* studier indikerede, at mikrocontainerne ville kunne beskytte vaccinen mod de barske forhold i mavetarm kanalen og levere den sikkert til tarmvæggen. Desværre forbedrede de ikke virkningen af cubosomes med OVA og Quil-A *in vivo* efter oral administrering. Resultaterne indikerer, at orale vaccineleveringssystemer bør benyttes til at forbedre virkningen af vacciner som har en vis effekt oralt, frem for at muliggøre oral levering af vacciner der er ineffektive oralt.

PUBLICATIONS

Paper I **Spray dried cubosomes with ovalbumin and Quil-A as a nanoparticulate dry powder vaccine formulation**

Christoffer von Halling Laier, Blake Gibson, Marco van de Weert, Ben J. Boyd, Thomas Rades, Anja Boisen, Sarah Hook, Line Hagner Nielsen

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Paper II **Design of experiments on spray drying parameters for producing cubosome precursors**

Christoffer von Halling Laier, Tommy Sonne Alstrøm, Mia Travers Bargholz, Pernille Bjerg Sjøltov, Thomas Rades, Anja Boisen, Line Hagner Nielsen

Technical note submitted to the European Journal of Pharmaceutics and Biopharmaceutics

Paper III **Microcontainers for protection of oral vaccines, *in vitro* and *in vivo* evaluation**

Christoffer von Halling Laier, Blake Gibson, Jorge Alberto S. Moreno, Thomas Rades, Sarah Hook, Line Hagner Nielsen, Anja Boisen

Manuscript in preparation (planned for submission to the Journal of Controlled Release)

CONTRIBUTIONS TO THE PAPERS

Paper I I designed, planned and executed the experiments with help from Blake Gibson and Sarah Hook for parts of the *in vivo* studies, Ben Boyd for the small angle x-ray studies and Marco van der Weert for the circular dichroism studies. I performed the data treatment and interpretation and wrote the paper.

Paper II I designed the study and the experiments. Line Hagner Nielsen and I supervised master students Mia Travers Bargholz and Pernille Bjerg Sjøltov who performed the experiments and did the initial data treatment. I performed the final data treatment except the Matlab coding which was done by Tommy. I interpreted the data and wrote the paper.

Paper III I designed and planned the experiments. I executed the experiments with help from Blake Gibson and Sarah Hook for parts of the *in vivo* studies and from Line Hagner Nielsen for the SAXS studies. Jorge Sevilla Moreno produced the vaccines for one of the oral vaccine studies. I performed the data treatment and interpretation and wrote the paper.

POSTER PRESENTATIONS AND OTHER WORKS

I. Spray Drying of Cubosomes for Oral Vaccine Delivery

Christoffer von Halling Laier, Ingrid Elise Konow Weydahl, Thomas Rades, Line Hagner Nielsen, Anja Boisen

2016 Annual Meeting and Exposition, American Association of Pharmaceutical Scientists, November 13-17 2016, Denver, USA.

(POSTER)

II. Spray dried cubosomes as effective vaccine delivery system

Christoffer von Halling Laier, Blake Gibson, Thomas Rades, Sarah Hook, Anja Boisen, Line Hagner Nielsen

2018 Controlled Release Society Annual Meeting and Exposition,
July 22-24 2018, New York City, USA

(POSTER)

III. Powder embossing method for selective loading of polymeric microcontainers with drug formulation

Zarmeena Abid, Carsten Gundlach, Onur Durucan, Christoffer von Halling Laier, Line Hagner Nielsen, Anja Boisen, Stephan Sylvest Keller

Microelectronic Engineering 171 (2017) 20–24

(Full paper)

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LIST OF SYMBOLS AND ABBREVIATIONS

AS01	Adjuvant system 01
APC	Antigen presenting cell
BCR	B cell receptor
CAF04	Cationic Adjuvant Formulation 04
CPP	Critical packing parameter
CTL	Cytotoxic T lymphocytes
cryo-FESEM	Cryogenic field emission scanning electron microscopy
Cryo-TEM	Cryogenic transmission electron microscopy
D	Diamond cubic structure
DBS	Dibutyl sebacate
DC	Dendritic cell
DOE	Design of experiments
DTP	Diphtheria-tetanus-pertussis
EL100-55	Eudragit® L100-55
FAE	Follicle-associated epithelium
G	Gyroid cubic structure
GI-tract	Gastro intestinal tract
H_1	Type 1 hexagonal phase
H_2	Type 2 hexagonal phase
Ig	Immunoglobulin

IPMS	Infinite periodic minimal surfaces
ISCOMs	Immune stimulating complexes
L_α	Laminar phase
MALT	Mucosa-associated lymphoid tissue
manLCP	mannosylated lipid core peptide
MCV	Measles containing vaccine
MHC	Major histocompatibility complex
M cells	Microfold cells
MMG	Monomycoloyl glycerol
MW	Molecular weight
OVA	Ovalbumin
P	Primitive cubic structure
PAMP	Pathogen-associated molecular pattern
PDI	Polydispersity index
pIgR	Polymeric immunoglobulin receptor
PP	Peyer's patch
PLGA	Poly(lactic-co-glycolic) acid
PRR	Pattern-recognition receptor
sIgA	Secretory immunoglobulin A
SAXS	Small angle x-ray scattering
s.c.	Subcutaneous
SEM	Scanning electron microscopy

TCR	T cell receptor
TLR	Toll-like receptor
V_1	Type 1 cubic phase
V_2	Type 2 cubic phase
VLPs	Virus like particles
WHO	World Health Organisation

TABLE OF CONTENTS

1. Introduction

In the 18th century in Europe, smallpox affected all layers of society and was responsible for an estimated 10 % of all deaths. Many of those that survived were left with disfiguring scars and were often blinded [1–3]. While it had been known for millennia that survivors of a smallpox infection became immune to the disease, no cure had been found. The most successful means of fighting smallpox was the process of subcutaneously injecting a small amount of smallpox virus from pus isolated from infected individuals into non-immune people. This process was called inoculation (from *inoculare* – to graft) or variolation (from *variola* – smallpox) [2,3]. The philosophy was that the injection of a small amount of virus generally would not lead to detrimental disease but could protect against natural infection occurring from inhalation of uncontrolled amounts of virus [2]. Variolation had long been used in China and India by the time it was introduced in Europe in the 18th century [1]. While variolation was effective, it carried the risk of causing smallpox disease and transferring other infections to the recipient. Variolated subjects also sometimes conveyed natural smallpox infection to susceptible individuals in their surroundings [1,2]. Variolation was a risky procedure associated with a variolation induced fatality rate of 0.5-2 %, but it gave about 80 % protection against natural infection. Since the fatality rate of people naturally infected with smallpox was around 20-30 %, many chose variolation believing that the risk of dying from it was smaller than the risk of dying from natural infection with the virus [1,4].

By the end of the 18th century, based on observations of milk maids with a history of cowpox resisting variolation or smallpox infection, Edward Jenner hypothesised that cowpox infection could (i) protect against smallpox and (ii) be deliberately transferred between people in order to infer protection against smallpox [1,3]. In May 1796, Jenner injected an 8 year old boy, James Phipps, with matter from fresh cowpox lesions on a milkmaid. Phipps developed light disease, but recovered and was later challenged with injection of fresh matter from a smallpox lesion. Since Phipps was unaffected, Jenner concluded that Phipps was protected. Jenner named the process vaccination (from *vaccinia* – cowpox), distinguishing it from variolation [1–3]. Louis Pasteur later devised the principle of isolating, inactivating and injecting the disease causing organism to induce protective immunity on which subsequent vaccines were based for many years [5]. In

honour of Jenner, he generalized the use of the term “vaccination” to include the preventive inoculation of any infectious agent [1].

Today, a number of vaccines are routinely administered in many countries, primarily to children and additional vaccines are often administered to people at risk, e.g. through travel to endemic regions [6,7]. Vaccines constitute the most significant medical contribution to public health and their positive impact on global human health is second only to clean drinking water, sanitation and hygiene [5,8,9]. The global eradication of smallpox after an extensive global vaccine effort organised by the World Health Organisation (WHO) [10] is a striking example of what can be achieved with effective global vaccination. However, the control of an infectious organism through vaccination relies not only on the availability of an effective vaccine, but also on a high vaccine coverage within a population.

In 1974, the WHO initiated the Expanded Programme of Vaccination with the aim of ensuring that children worldwide have access to four routinely recommended vaccines: Bacillus Calmette-Guérin (protecting against tuberculosis), polio vaccine, measles containing vaccine (MCV) and the diphtheria-tetanus-pertussis (DTP) vaccine [11]. The program has been highly successful elevating the global coverage of the third dose of the DTP vaccine (DTP3 – often used as measure of vaccine coverage) from 5 % in 1974 to 83 % in 2011 [12]. Since then, however, global coverage of the first dose of MCV (MCV1) and DTP3 has been stagnant leaving many children unprotected from infections that could be avoided through vaccination. [11,13]. In 2016, eight countries had DTP3 coverage rates lower than 50 %. Nearly all of them were facing conflict or serious economic turmoil thus greatly complicating the maintenance of vaccine coverage [11].

Most vaccines need to be kept at 2-8ºC from manufacture to administration generating a logistic challenge referred to as the cold chain [14]. The vaccines are usually administered by injection by trained healthcare personnel [5]. In addition, most vaccines need to be given 2-3 times with months between them to provide protection, and this can be infeasible in areas with limited access to healthcare [15,16]. The DTP vaccine is recommended to be given three times to all children. Nonetheless, 14 % of children worldwide did not receive the DTP3 vaccination leaving them unprotected, even though 34 % of them had received at least one DTP vaccination [11]. It is therefore imperative for global vaccine coverage to develop vaccines which can easily and inexpensively be distributed and administered in areas with limited logistical and healthcare infrastructure.

Heat stable self-administrable vaccines would be able to greatly reduce the problems of logistics, costs and repeated healthcare visits. They may therefore substantially improve vaccine coverage. The most attractive route of administration is the oral route, e.g. with the vaccine in a capsule [5,17]. Heat-stable vaccines in capsules can be distributed easily and, since needles are obviated, people can self-medicate according to an instructed vaccination schedule. Needles constitute the main risk associated with vaccination in developing countries through improper use. They additionally generate a large biohazardous waste problem that many developing countries lack the infrastructure to handle properly [13,17]. Oral vaccines hence solve many practical challenges associated with injected vaccines. Another important benefit of oral vaccines is the potential to stimulate both mucosal and systemic immunity providing better protection than parenteral vaccines, which mainly induce systemic immunity [18,19]. Oral vaccines can elicit mucosal immunity in the gastrointestinal tract (GI-tract) and at distant mucosal surfaces [20–22], e.g. the rectal or vaginal mucosa [23].

Vaccines have traditionally been based on live attenuated or whole inactivated organisms which have a strong intrinsic immunostimulatory effect. However, these can have the risk of unacceptable side effects [5,24]. Modern vaccines are therefore based on highly purified antigenic fragments of pathogens, often peptides, instead of a weakened or killed form of the entire pathogen. These vaccines are referred to as subunit vaccines and have excellent safety profiles but are less immunogenic than whole organism vaccines. In order for them to be effective, they must therefore be co-administered with adjuvants – substances that improve the immunogenicity of the subunit antigen [5,17].

Subunit vaccines are particularly challenging to develop for oral delivery for reasons described in sections 2.2 “Biological barriers of the gastrointestinal tract” and 3.2 “Oral vaccines”. Some of the issues may be overcome with the use of advanced drug delivery systems as e.g. microcontainers, which have lately been proposed as a promising system to improve oral drug delivery of small drug compounds that are poorly soluble in water and/or have poor permeability across the intestinal epithelium [25–27]. Microcontainers are polymeric devices in the micro-meter size range (Chapter 5). Those used by Nielsen et al. [27] and Mazzoni et al. [25] as well as for this thesis are cylindrical reservoirs with height and diameter of approximately 300 µm and an opening at one end allowing drug loading

and drug release. These are thought to have the potential to allow oral delivery of subunit vaccines [28], but have never been thoroughly evaluated for this purpose.

1.1. Aim of this PhD project

The aim of this PhD project was to apply microcontainers as an oral delivery system for a subunit vaccine formulation. To achieve this, the microcontainers should ideally (i) contain a high load of vaccine formulation, (ii) be sealed with lids that protect the vaccine from chemical and enzymatic degradation in the stomach and (iii) deliver the vaccine to the microfold cells (M cells) in the intestine.

This implies a number of restrictions to the choice of vaccine. The ideal vaccine for use with microcontainers should (i) carry a high antigen load, (ii) be in a powder form that is stable at room temperature, (iii) be compatible with low-cost and high-throughput production and (iv) be effective at mucosal surfaces. Such vaccine properties can potentially be achieved using a spray dried subunit vaccine employing the inexpensive and mucosally active adjuvant Quil-A. The state of the art microcontainers developed in the Nanoprobes group at DTU Nanotech used together with a pH-sensitive polymer lid are potentially suitable to achieve the desired properties of the microcontainers. Therefore, this PhD project aimed to spray dry a vaccine formulation with Quil-A as adjuvant and ovalbumin as model antigen, load this into microcontainers and coat the loaded microcontainers with a pH-sensitive lid to deliver the vaccine safely through the stomach to the small intestine.

To achieve the goal of this PhD project, two major accomplishments needed to be achieved. First, a subunit vaccine platform was chosen, designed, produced and characterised *in vitro* and *in vivo*. Second, microcontainers needed to be tailored for oral vaccination of mice. This included enlarging the cavity of the microcontainers, establishing a method to load the powder vaccine into the microcontainers and designing a lid for targeted release in the small intestine of the mouse. Finally, the system was evaluated *in vivo*.

1.2. Structure of the thesis

This introductory chapter is followed by four chapters providing background for the PhD project. Chapter 2 gives an introduction to the immunology relevant for the PhD project. This is followed by a description of the biological barriers that need to be considered when designing an oral delivery system for vaccines and finally mucosal immunology. Chapter 3 gives a general introduction to vaccines and oral delivery of these. The use of subunit vaccines is motivated and important elements to make them effective, namely immunopotentiators and particle delivery systems, are introduced. Immunopotentiators used in this PhD project are presented and those considered to be the gold standard briefly introduced. Important design characteristics for particle-based delivery systems for subunit vaccines are then discussed followed by a presentation of selected state of the art particle systems. In Chapter 4, the formation, structure and characteristics of cubosomes is presented. This is followed by a description of typical lipids used to make non-lamellar liquid crystals, important characterisation methods and a description of different strategies to produce cubosomes. Finally, spray drying of cubosomes and its motivation is presented. Chapter 5 introduces the microcontainers used for oral delivery of the vaccines. The experimental work and results obtained during this PhD are presented and discussed in Chapters 6-8. Chapter 9 provides an elaborated general discussion of important results from Chapters 6-8 and additional unpublished data are included where relevant. Chapter 10 and 11 completes the thesis with general conclusions and future perspectives.

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2. Physiology and immunology

Vaccine design was entirely empirical in the days of Jenner and Pasteur. Since then, the science of immunology has developed greatly. The improved understanding of physiology and immunology along with the tools provided by other scientific progresses has paved the way for rational vaccine design [1] and improved preclinical experimental approaches to evaluate vaccine candidates [2–5]. In section 2.1 and 2.3, the immunology relevant for this thesis is presented, and in section 2.2, the physiology of importance to oral vaccination is presented.

2.1. Immunology

The immune system is often divided into two categories: the innate and the adaptive immune system. The innate immune response is fast responding (within minutes) and acts as the first line of defence but is generally not antigen specific [6]. It is comprised of physical barriers (e.g. the epithelium and mucus), soluble substances (e.g. the complement system) and phagocytic and antigen presenting cells (APCs) that recognize and interact with pathogens [7]. Innate immune cells recognize pathogens and distinguish them from self-antigens and non-pathogenic organisms via pattern-recognition receptors (PRRs). PRRs bind to pathogen-associated molecular patterns (PAMPs) expressed uniquely by pathogens [6]. Toll-like receptors (TLRs) are the most extensively described family of PRRs and agonists of these receptors have been shown to be potent vaccine adjuvants [8]. The cells of the innate immune system respond to PRR stimuli by inducing inflammation and recruiting the adaptive immune system to eradicate the infection [6].

Most pathogenic organisms are dealt with by the innate immune system. When the innate immune system is overwhelmed by a pathogen, an adaptive response is stimulated. The adaptive immune response is specific to a specific pathogen and is based on recognition of unique epitopes on an antigen from the pathogen rather than general PAMPs. The adaptive immune response to a specific pathogen is acquired during the first exposure to it (the primary immune response). It takes longer to develop than the innate response, but is much more potent. After resolution of the infection, memory cells remain in the body allowing a strong antigen-specific immune response to be elicited fast upon later exposure to the same

antigen. This provides immunity to pathogens carrying the antigen [6]. The adaptive immune response is divided into two categories based on the effector mechanism of the response: the humoral and the cellular immune responses. The humoral immune response is mediated by antibodies and the cellular immune response is facilitated by cytotoxic T cells. The response elicited depends on how the antigen is presented by the APC, which in turn is determined by whether the antigen is present in the cytosol or in the endosomal or extracellular compartment (Figure 1) [9].

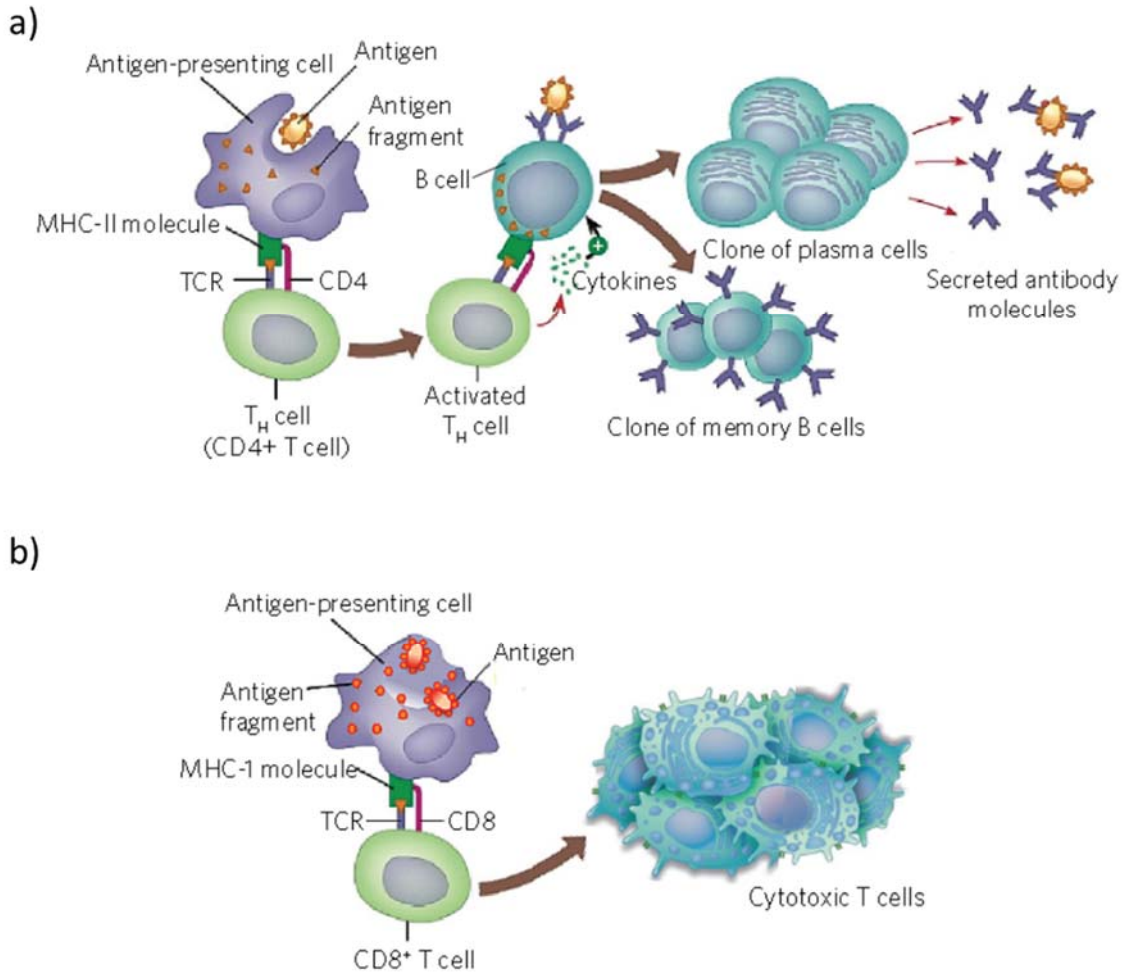


Figure 1. Antigen presentation and T cell activation by antigen presenting cells. a) Extracellular antigens are internalised by APCs, broken down into peptides and presented on the surface of the APC in the context of MHC-II molecules. $CD4^+$ T cells, also known as T helper or T_H cells, recognise the MHC-II/peptide complex through their T cell receptor (TCR) and $CD4^+$ surface molecules. The activated $CD4^+$ T cell can then provide help for other cells specific to the same antigen epitope, as exemplified in the illustration by activation of B cells to become antibody secreting plasma cells and memory cells. b) Intracellular antigens are broken down into peptides and loaded onto MHC-I. The MHC-I/peptide complex is transported to the surface of the APC where it can be recognised by $CD8^+$ T cells through their TCR and CD8 surface molecules. Activated $CD8^+$ T cells proliferate and mature into cytotoxic T cells capable of killing other cells that present the same antigen/MHC-I complex on their surface. Modified from [10] *with permission from Springer, Nature Chemical Biology, Copyright 2013.*

Extracellular pathogens are taken up through phagocytosis and broken down to short peptides (typically 13-22 amino acids in length) in lysosomes by low pH and enzymes in a process known as antigen processing. These peptides are subsequently complexed with Major Histocompatibility Complex (MHC) II (MHC-II) molecules and presented on the surface of APCs for recognition by $CD4^+$ T cells (described below) in a process referred to as antigen presentation (Figure 1a) [9,11]. MHC-II is mostly expressed by specialized immune cells such as macrophages, dendritic cells (DCs) and B cells [12].

Intracellular pathogens (e.g. intracellular virus) are broken down by the proteasome complex in the cytosol creating short peptide antigens (often 8-10 amino acids). These are complexed with MHC-I molecules and presented on the surface of the cell for recognition by cytotoxic $CD8^+$ T cells (described below). All nucleated cells are capable of expressing MHC-I molecules with antigen on their surface and can thereby be recognized and lysed by cytotoxic $CD8^+$ T cells if they have been infected (Figure 2). However, in order for this to happen, naïve $CD8^+$ T cells must first be activated by DCs to mature effector cytotoxic $CD8^+$ T cells [9,13], as illustrated in Figure 1b.

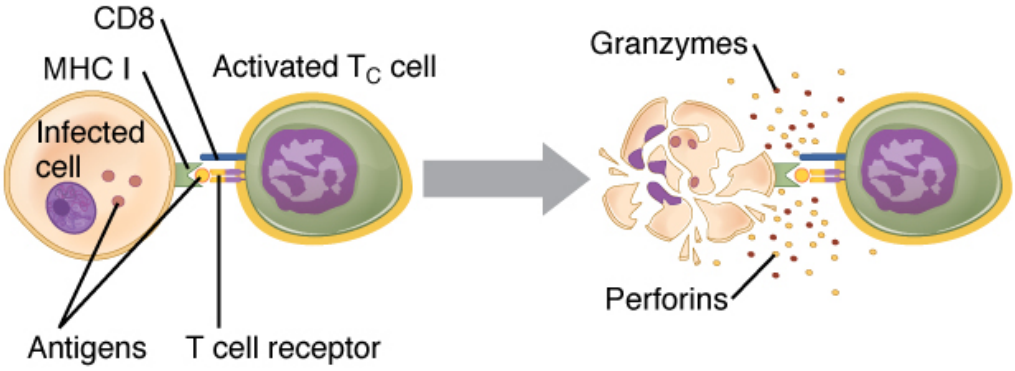


Figure 2. All nucleated cells are capable of expressing MHC-I. When a cell is infected by a pathogenic organism, it presents antigens from the organism on its surface in the context of MHC-I. This allows cytotoxic $CD8^+$ T cells to identify and kill infected cells without attacking healthy cells. Granzymes and perforins are examples of cytotoxic effector molecules produced by cytotoxic $CD8^+$ T cells. Modified from [14].

DCs are present in an immature state in all parts of the body and have been recognized to play a central role in stimulation and regulation of adaptive immunity. Immature DCs sample antigens through phagocytosis, micropinocytosis and receptor mediated endocytosis. DCs mature upon activation by stimulation of PRRs, migrate to draining

lymph nodes and upregulate surface expression of co-stimulatory molecules. They serve as potent APCs in the peripheral lymphoid organs (lymph nodes, spleen and mucosal lymphoid tissues) and are the only cells able to stimulate activation of naïve $CD8^+$ T cells to become effector $CD8^+$ cytotoxic T cells. Internalised antigens can be presented by DCs on MHC-I molecules in a process called cross-presentation. DCs regularly present the same antigen on both MHC-I and MHC-II molecules and most pathogens therefore initiate both a $CD4^+$ and a $CD8^+$ response [9,15,16].

Lymphocytes of two types comprise the effector cells of the adaptive immune response: B cells and T cells. Naïve lymphocytes travel with the blood to circulate the peripheral lymphoid organs and return to the blood via the lymphatics to re-circulate. The peripheral lymphoid organs are the sites where adaptive immune responses are initiated through frequent meetings between lymphocytes and APCs. T cells interact only with processed antigen bound on MHC-I or MHC-II on the surface of APCs (Figure 1). Once the naïve T cell meets its specific antigen in the context of an MHC molecule and is activated by co-stimulatory molecules, it proliferates and differentiates into effector T cells. If co-stimulation is absent, the T cell will in contrast develop anergy leading to tolerance (illustrated for a $CD4^+$ cell in Figure 3). Effector T cells are classed based on surface proteins into $CD4^+$ and $CD8^+$ T cells [16,17].

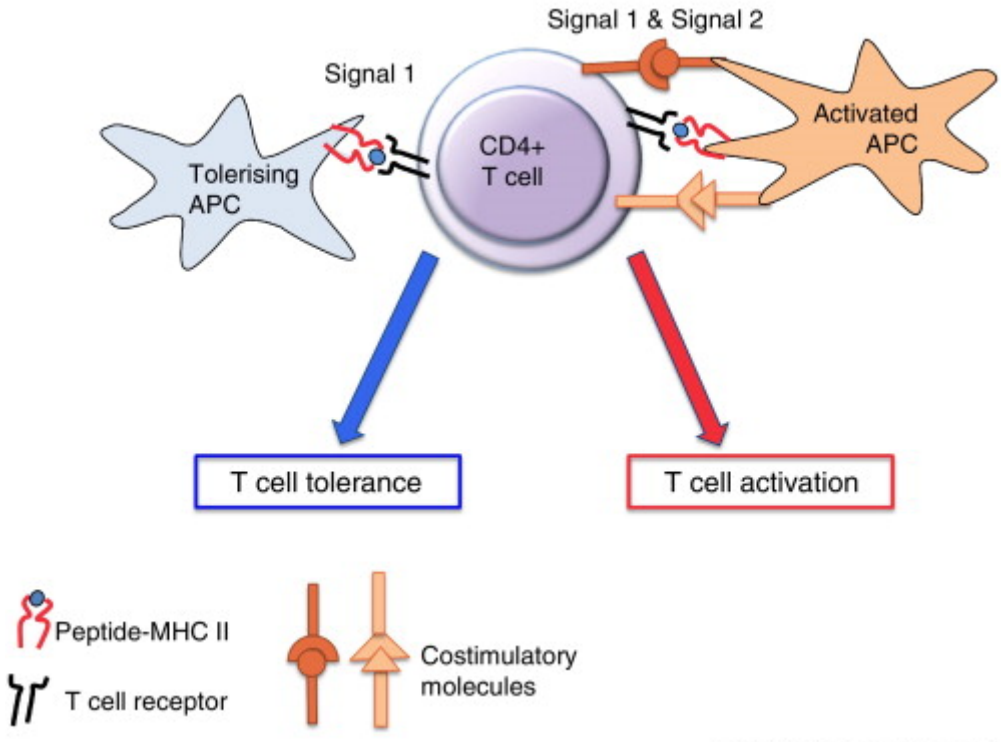


Figure 3. T cell activation requires presentation of antigen on MHC molecules (signal 1) and activation by co-stimulatory molecules (signal 2), as illustrated here for CD4$^+$ T cell activation by an MHC-II/peptide complex. Without co-stimulation, T cells will develop anergy leading to tolerance. Reprinted from [18].

CD4$^+$ effector T cells are also known as T helper cells and have a wide range of functions that help orchestrate, strengthen and regulate the immune response. CD4$^+$ T cells can for example stimulate B cells to become antibody secreting plasma cells (Figure 2a), amplify DC activation of CD8$^+$ T cells or boost the activity of macrophages. CD4$^+$ T cells can differentiate into several subsets of functional classes depending on their activation. The main classes are T_H1, T_H2, T_H17, T_{FH} and T_{reg}, each providing help and regulation for different functions of the immune system [16].

CD8$^+$ effector T cells are known as cytotoxic T lymphocytes (CTLs) and are important for protection against intracellular infections and cancers. Mature CTLs travel from the secondary lymphoid organs they were derived from to sites of infection. Here, they mediate pathogen clearance by killing infected cells and secreting chemokines and cytokines that recruit and activate other effector cells such as neutrophils and macrophages. Infected cells are distinguished from healthy cells by the CTL, through display on their surface of MHC-I with the antigen specific to the CTLs T cell receptor (Figure 2). After resolution of the

infection, 90-95 % of the CTLs undergo apoptosis leaving 5-10 % behind as a long-lived population of memory cells [5,19]. Generation of a CTL response can occur through activation by DCs on their own or with help (licensing) from $CD4^+$ T cells. $CD8^+$ T cell priming by DCs without $CD4^+$ T cell help is typically observed from infections associated with strong inflammation [5,20]. In the absence of overt inflammatory signals, $CD4^+$ T cell help is usually required to enable DCs to stimulate a CTL response. $CD4^+$ T cell help is additionally crucial for the generation of long-lasting effective $CD8^+$ T cell memory irrespective of the presence of inflammation during priming. While CTL responses generated without $CD4^+$ T cell help often are effective in clearing the infection, the resulting memory population tends to be defective in its ability to generate effective recall responses following secondary exposure to the pathogen (Figure 4) [5,20,21].

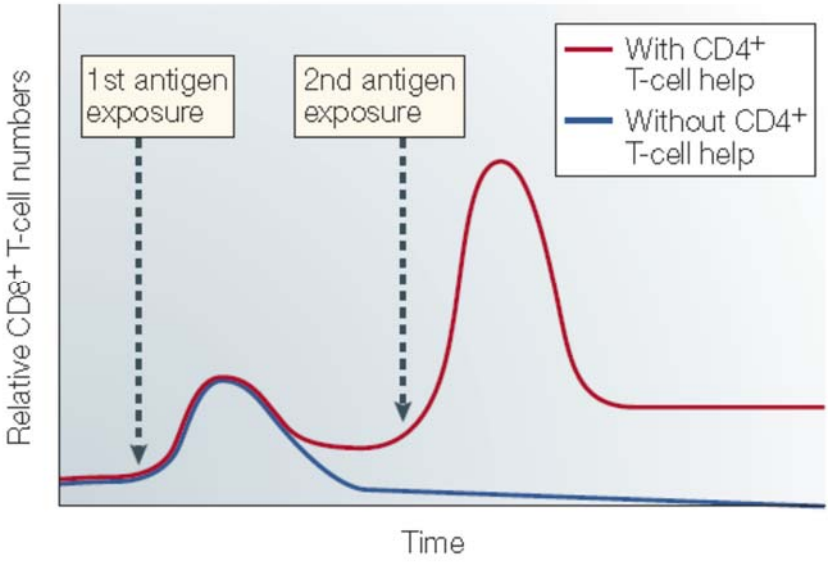


Figure 4. $CD4^+$ T cell help is required for the generation of long-lasting effective $CD8^+$ T cell memory. The graphs depict the magnitude of the CTL response to cellular non-inflammatory antigen. The primary response is weak and independent of $CD4^+$ T cell help. However, only with $CD4^+$ T cell help are long-lived functional memory cells created and thus the strong secondary response depends on $CD4^+$ help. Reprinted from [22] *with permission from Springer, Nature Immunology Reviews, Copyright 2004.*

In contrast to T cells, B cells interact directly with the native soluble antigen through their transmembrane immunoglobulins (Ig) known as B cell receptors (BCRs). Secreted Ig of the same specificity as the BCR are known as antibodies and are important for protection against extracellular pathogens and toxins. Upon activation by antigen, B cells proliferate and differentiate into antibody secreting plasma cells and memory B cells. Plasma cells can

produce 5 classes of antibodies: IgA, IgD, IgE, IgG and IgM (briefly presented below), but each cell produces only antibody specific to a single antigen [23,24]. Each class has different effector functions and biological properties [24,25].

Naïve but mature B cells exclusively express IgM and IgD on their cell surface [24,25]. B cell activation by antigen usually requires help from already activated $CD4^+$ T cells specific to the same antigen, but they can also be activated independently of $CD4^+$ T cells. Some microbial constituents, for example, that contain multiple repeating antigenic epitopes can activate B cells in the absence of $CD4^+$ help. After activation, B cells undergo immunoglobulin isotype switching to produce IgG, IgE or IgA, and affinity maturation in which their DNA is rearranged to produce antibodies with higher affinity to the antigen. The antibody isotype that is produced depends on the type of T cell response occurring in response to the pathogen [26,27]. IgM antibody is expressed early in the B cells development before extensive affinity maturation has occurred. It is therefore less specific than other isotypes allowing quick IgM antibody responses to a variety of antigens. IgM functions mainly by opsonising antigen and activating complement. IgD antibody is expressed in serum at very low levels and its function remains unclear. IgE is important for immune defence against parasitic infections but is also associated with hypersentivity and allergy. IgG is the predominant isotype in serum and extracellular fluid with the longest serum half-life. It functions by activating the complement system, opsonising or neutralising antigen, and establishes antibody dependent cytotoxicity by activating effector cells such as natural killer cells to destroy the antibody coated antigen. IgA is the principal class in mucosal secretions (section 2.3), but exists also in the blood [24,27].

2.2. Biological barriers of the gastrointestinal tract

The main purpose of the GI-tract is absorption of nutrients and water from ingested materials. The human GI-tract consists of the mouth, pharynx, esophagus, stomach, small intestine, large intestine and anus (Figure 5). The small intestine is mainly responsible for nutrient absorption and is functionally divided into the duodenum (where bile and pancreatic secretions are introduced), jejunum and ileum. The large intestine is divided into the caecum, colon, rectum and anal canal [28]. The human GI-tract has a large surface area to accommodate sufficient absorption of nutrients and water [29]. Reports of the total surface area of the GI-tract traditionally range from 260-400 m^2, but 30-40 m^2 has more

recently been suggested [30,31]. The surface area of the small intestine accounts for more than 90 % of the total area of the GI-tract [31]. The large surface area is heavily exposed to ingested infectious organisms [29] and it is therefore equipped with physical and biological barriers to protect against constant infection [32]. For the purpose of this thesis, we shall restrict the discussion to the segments of the GI-tract most relevant to oral delivery of vaccines: the stomach, the small intestine and, for the case of some vaccines, the colon. Common to the three segments is that they are lined by an epithelium and coated with mucus. The main cell type of the epithelium is the absorptive enterocytes followed by mucus secreting goblet cells [33]. The epithelial cells are connected by tight junctions making the paracellular space impermeable [34,35]. Their apical surface is covered by a carbohydrate coat created by integral plasma membrane glycoproteins and glycolipids. This coat is known as the glycocalyx [36]. Below the epithelial layer is a layer of connective tissue (*lamina propria*) and then a layer of smooth muscle cells (*muscularis mucosae*) [33].

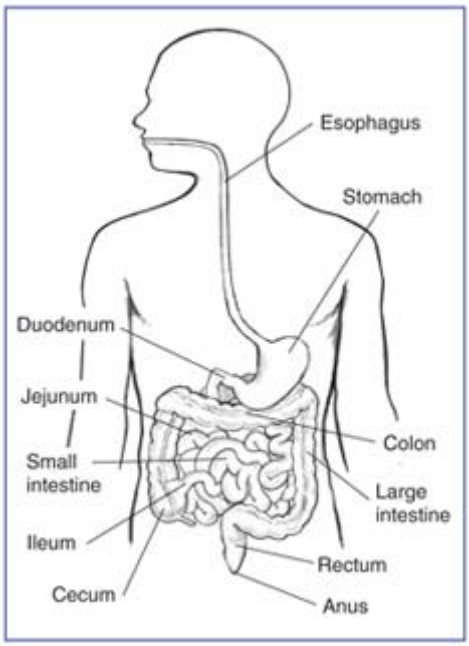


Figure 5. Anatomy of the human GI-tract. The GI-tract starts at the mouth and continues through the pharynx, esophagus, stomach, small intestine (duodenum, jejunum and ileum), the large intestine (caecum, colon, rectum and anal canal) and finally the anus. Reproduced from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)[37].

The main function of the stomach is to store and release food slowly into the intestine. Secretion of hydrochloric acid acidifies the stomach to a pH range of 1.0-2.5 in humans [34], although it may increase to around pH 5 after ingestion of a meal [38]. The low pH

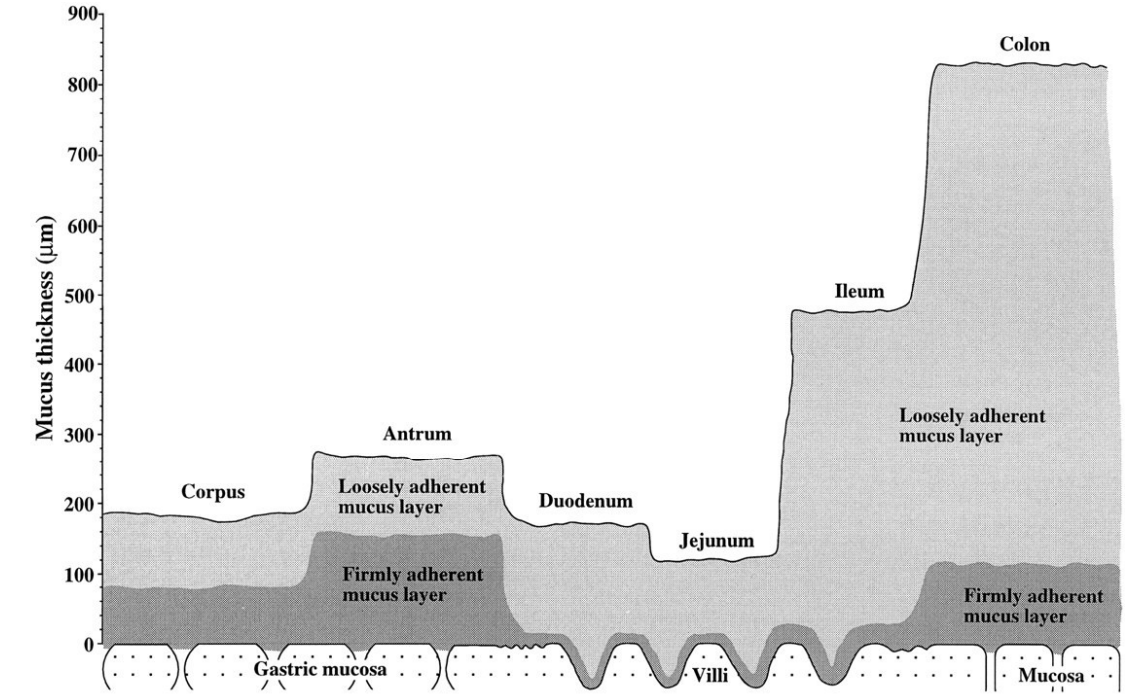
chemically degrades food components and inactivates many ingested organisms [34]. The epithelial cells also secrete the proteinase pepsin, which is active at low pH and hydrolyses proteins into smaller peptides [35]. The epithelium in the stomach is lined by a thick mucus layer (40-450 μm) into which bicarbonate is secreted to neutralise the pH locally and inactivate pepsin. This separates the harsh luminal milieu from the epithelium thus preventing self-digestion [34].

In the small intestine, the pH ranges from 6.2 proximally to 7.4 distally [39]. Bile and pancreatic secretions are added to the food chyme at the proximal end of the duodenum. The bile and bicarbonate ions from the pancreatic secretions quickly bring the pH of the acidic chyme from the stomach up towards neutral pH. Bile salts emulsify fats and pancreatic enzymes break down proteins, lipids and carbohydrates for absorption. This forms a significant barrier for subunit antigens [28,34]. A continuously replaced mucus layer covers the epithelium of the small intestine and effectively traps and clears pathogens and foreign particulates [30,40].

The mucus of the GI-tract is generated mainly by negatively charged glycoproteins called mucins, which entangle and cross-link adhesively and reversibly to form a dynamic viscoelastic gel [30]. Mucins can be divided into two classes: secreted gel forming mucins present in the mucus and cell bound mucins present in the glycocalyx [30,41]. Mucus consists mainly of 95-99 % water and 5-1 % glycoproteins, but it also contains lipids, carbohydrates, salts and other substances [30,42]. The gel forms a mesh which can sterically block the entrance of particles larger than a certain size. It contains hydrophilic regions of heavily glycosylated mucins and regions of naked proteins. Lipids adsorbed to naked protein domains compose 20 % of the dry weight of the mucus and generate a hydrophobic adhesive barrier making it difficult for hydrophobic substances to cross the barrier [30].

Mucus in the GI-tract is present in two layers: a firmly adherent layer in connection with the glycocalyx and an overlying loosely adherent layer [33]. However, there is disagreement about whether the small intestine has both layers of mucus or only a layer of loosely adherent mucus [33,41]. The mucus layers have varying thickness through the GI-tract, as illustrated in Figure 6 from measurements in rats [43]. In mice, the mucus appears to be thinner and vary differently according to the segment of the GI-tract than the thicknesses indicated in rats in Figure 6: in the ileum of C57Bl/6 mice (the strain used

throughout this PhD), the mucus thickness is approximately 210 µm [44] and in the distal colon it is approximately 125-150 µm [44,45]. Below the mucus layer is the glycocalyx of the epithelial cells (Figure 7). In contrast to the mucus, the glycocalyx is anchored to the cell membrane by transmembrane mucins. The glycocalyx of the enterocytes is approximately 0.5-1.5 µm thick and provides a dense barrier with properties similar to the mucus [41].



Mean mucus thickness (µm)

Total	189 ± 11	274 ± 41	170 ± 38	123 ± 4	480 ± 47	830 ± 110
Firmly Adherent	80 ± 5	154 ± 16	16 ± 3	15 ± 2	29 ± 8	116 ± 51
Loosely Adherent	109 ± 12	120 ± 38	154 ± 39	108 ± 5	447 ± 47	714 ± 109
n	6	6	7	6	5 (8 firm)	11

Figure 6. Schematic of the thickness of the two layers of mucus in the GI-tract of the rat: the firmly adherent mucus closest to the epithelium and the loosely adherent epithelium closest to the lumen. The mucus layer is continuous and does not follow the contours of the villi in the intestine. The table shows the measured mean thickness (µm) and standard error of the two layers of mucus. Figure reprinted from [43].

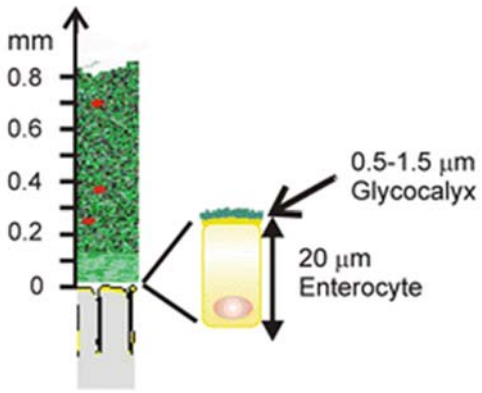


Figure 7. Schematic showing the relatively thin glycocalyx coating of enterocytes under the mucus layer in the distal rat colon. Figure adapted from [41] *with permission from Springer Nature, copyright 2011.*

The colon has been reported to have a pH of 6.6±0.7 with increasing pH from the proximal to the distal end [46]. It has lower enzymatic activity than the small intestine [34] and is covered by a much thicker mucus layer [30]. The colon further contains a large commensal microbiota that benefit the host by supplying nutrients, digesting foods that the host cannot digest on its own and helps prevent colonization by harmful organisms [32].

The digestive function of the GI-tract and the presence of the commensal microbiota give rise to additional biological barriers for oral vaccination. Oral vaccines face dilution over the large surface area of the GI-tract and by mucosal secretions thus requiring large doses [47]. Repeated ingestion of large antigen doses, however, may lead to the generation of tolerance rather than immunity [48]. Tolerance is defined as suppression of the immune response to specific antigens and is important for avoiding that beneficial microorganisms and food substances are attacked by the immune system [32]. Soluble proteins are generally only taken up in small amounts and tend to induce immune tolerance [49] thus necessitating that oral vaccines express appropriate danger signals to trigger an immune response [35].

For evaluation of oral vaccine delivery systems in mice, it is important to consider the differences of the GI-tract between humans and mice. The GI-tract of mice is composed of organs that are anatomically similar to those of humans although prominent differences exist, as illustrated in Figure 8. The large difference in body size obviously leads to large size differences of the organs. Other differences might be related to different diets, feeding patterns and metabolic requirements [29,50]. The stomach of some mouse strains is divided

into a non-glandular forestomach and a glandular part. The small intestine in the mouse does not have the mucosal folds (*plicae*) of the human small intestine, but has villi taller than those of humans to increase its surface area [50,51]. The small intestine to large intestine length and surface area ratios in humans are 7 and 400 while they are only 2.5 and 18 in mice [50]. The caecum in mice is relatively large and is an important site for fermentation of plant materials and vitamin production, which mice reabsorb through coprophagy. The human caecum is, in contrast, relatively small, has no fermentation and is without a clear function. The thickness of the mucus in the mouse colon is thinner than that of humans and 85 % of the large intestinal microbiota of mice are not present in humans [50].

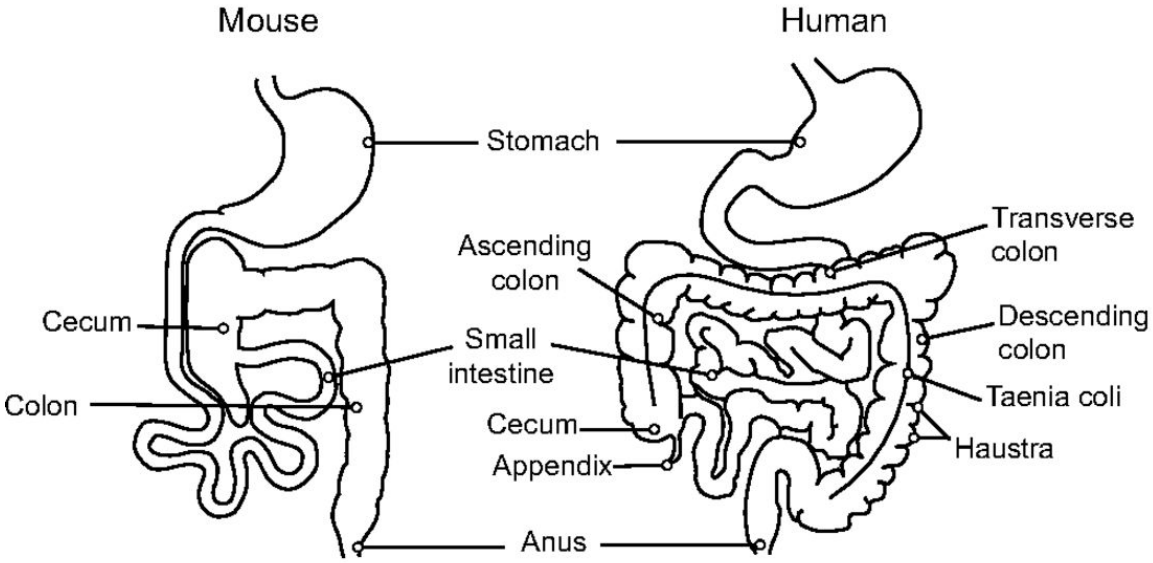


Figure 8. Anatomy of the GI-tract in mice and humans. The relative sizes and lengths of the different segments of the GI-tract are quite different between man and mouse. Humans have a relatively larger small intestine and mice a relatively larger caecum and colon. Reprinted from [50].

Immunological differences are also present. Paneth cells are present in the small intestine of both species where they secrete antimicrobial compounds into the lumen. In mice, these cells are also present in the caecum but absent in the colon whereas they are present in small amounts in the colon of humans [50]. Peyer's Patches are important sites for the induction of immune responses as described in section 2.3. The amount, size, distribution and composition of these vary between species and sometimes strain [52].

Of obvious importance to the evaluation in mouse models of oral drug and vaccine delivery systems that employ a pH-controlled release mechanism, are the differences between man and mouse in transit times and pH-ranges through the GI-tract. The pH of the stomach and small intestine of mice has been reported to vary between different strains of mice [51]. The pH of the stomachs of the common laboratory mouse strain Balb/c has been reported to be 3.0±0.3 in the fed state and 4.0±0.2 in the fasted state [53] thus being higher than what is generally reported in humans and oppositely affected by fasting than the pH of the human stomach. The pH of the small intestine of Balb/c mice was reported to be lower than in humans with a pH just below 5 in the small intestine of fed mice [53]. In C57Bl/6 mice (the strain used for our studies), the pH was reported to be 6.7 in the ileum [54] and thus closer to that of humans.

The transit time through segments of the GI-tract is highly variable. In humans, gastric emptying time is often reported to range from 5 min to 2 h [39]. Transit times through the small intestine are often 2-4 h [55] and through the large intestine typically 6-32 h [39]. In a study by Padmanabhan et al, mice were orally gavaged with a radioactive label and visualised at various time-points showing labelling of the stomach and small intestine within 70 min, and of the entire GI-tract within 6 h [56]. This indicated that the transit time through the mouse GI-tract is much shorter than in humans as would be expected due to its much shorter dimensions and the relatively constant feeding pattern of mice. In Paper III (Chapter 8), the transit kinetics of microcontainers through the mouse GI-tract was investigated and showed that microcontainers reach the caecum within 60 min of administration and that more than 60 % of the number of administered microcontainers passed the small intestine within 90 min. Oral delivery systems for use in mice thus need quick release times which might not be optimal for humans. A further consideration important for the safety of the mouse in connection with oral administration, is that mice are incapable of vomiting [57].

2.3. Mucosal immunity

Most pathogens enter the human body through mucosal surfaces [32,58], and mucosal immunity is therefore an important element in our immune system to keep infectious organisms at bay. Here, the meaning of mucosal immunity shall be restricted to the protection inferred at mucosal surfaces by the adaptive immune system and focus will be

on the GI-tract. The mucosal immune system is divided functionally and anatomically into the organised mucosa-associated lymphoid tissues (MALTs) and the diffuse *lamina propria* region and glandular tissues. The MALT is responsible for initiating immune responses and the *lamina propria* and glandular tissues are effector sites for humoral and cellular immune responses. The constant migration of antigen-activated immune cells between inductive and effector sites provides the basis for the common mucosal immune system [32]. The common mucosal immune system forms a network that makes it possible for immunisation at one mucosal site to provide immunity at other mucosal sites, as illustrated in Figure 9 [58]. However, the MALT is divided into anatomically distinct compartments and functions essentially independently of the systemic immune system [59]. There is also functional compartmentalisation of the MALT in the way that specific sites of induction tend to stimulate strong immune reactions in some but not all of the effector sites [59]. Orally administered vaccines can stimulate effector responses locally as well as at a wide range of distant mucosal sites (Figure 9) [59]. Oral vaccination may thereby allow for immunisation at distant mucosal sites that are less practical for vaccine administration.

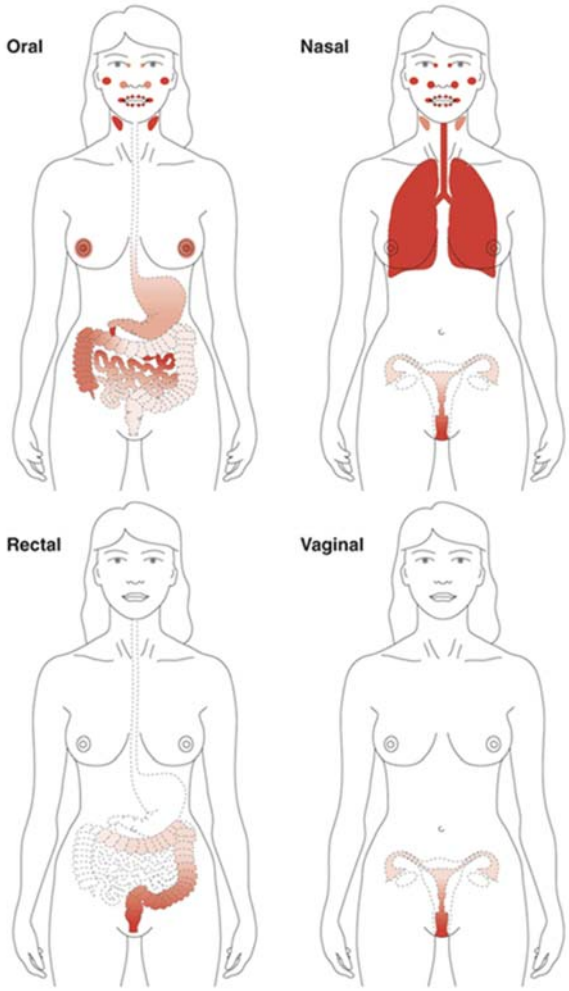


Figure 9. Illustration of the common mucosal immune system and its compartmentalisation. The red shading indicates expression of mucosal IgA immune responses after vaccination by the oral, nasal, rectal or vaginal route. The strength of the response is indicated by the strength of the colour shading. The strongest response is generally seen at the site of immunisation and adjacent mucosae. However, oral and nasal vaccination can give strong immune responses at distant mucosal sites. Reprinted from [59] *with permission.*

The epithelial cells of the GI-tract express a polymeric Ig-receptor (pIgR), MHC-I and -II and a range of secretory molecules and adhesion molecules [58]. The *lamina propria* beneath the epithelium is a major effector site of mucosal immunity and is host to B cells, T cells, macrophages and other immune cells [60]. Along the length of the small intestine are macroscopic lymphoid tissues known as Peyer's Patches (PPs) in which DCs, B cells and T cells reside [29], as illustrated in Figure 10.

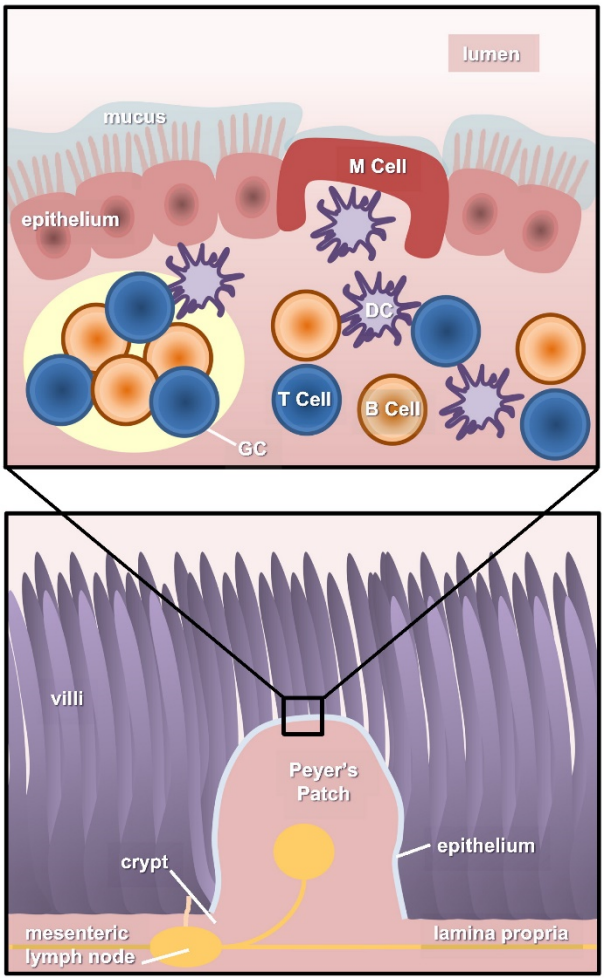


Figure 10. Schematic of the anatomy of the gastrointestinal mucosal immune system. It consists of two major components, namely the effector sites and the inductive sites. The effector sites comprise the lamina propria and the epithelium; the inductor sites comprise the PP's, lymphoid follicles and dispersed APCs. PP's are macroscopic lymphoid tissues containing APCs, B cells and T cells and are covered by follicle associated epithelium (FAE) in which M cells reside. Reprinted from [35] *with permission.*

Dendritic cells residing in the *lamina propria* of the GI-tract can extend their dendrites past the epithelium and into the lumen thus allowing them to sample passing antigens [61]. Another important mechanism of antigen sampling by APCs is mediated by special epithelial cells called Microfold cells (M cells). M cells are present in the follicle-associated epithelium (FAE) in the intestine (e.g. lining the PPs). They take up organisms and particles smaller than 10 µm from the lumen of the intestine and transport them across themselves to the underlying APCs (Figure 10 and Figure 11) [62,63]. A deep invagination on the basolateral membrane of the M cell forms an extracellular compartment in the M cell where macrophages, DCs, B cells and T cells reside (Figure 11). Uptake of particles from the

intestine is followed by quick transcytosis of the intact particle to the APCs behind the M cell resulting in antigen processing and presentation [60,64,65]. M cells appear to be the main route of entry into the host for several enteric pathogens such as polio, human immunodeficiency virus and *salmonella typhi* [64,66] and are thus appealing vaccine targets. Uptake by M cells generally does not result in degradation and so antigens and microorganisms are generally transcytosed intact/alive across M cells to the underlying lymphoid tissue [60,64].

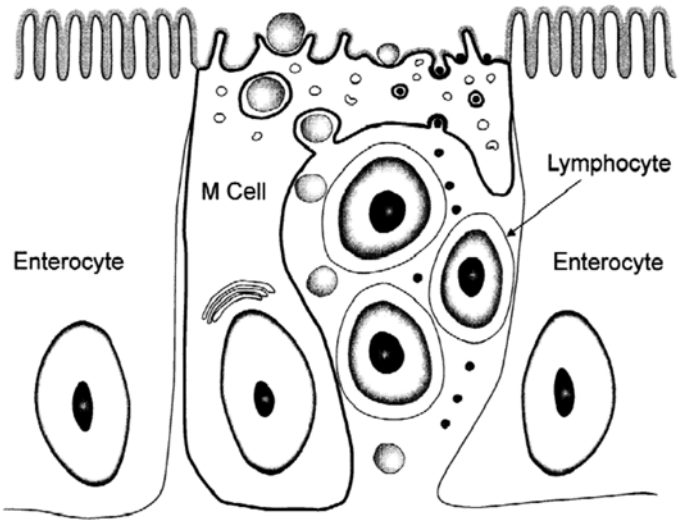


Figure 11. Schematic of an M cell in follicle associated epithelium. M cells are covered with a thinner mucus than normal enterocytes and have short irregular microvilli allowing close contact with antigens in the intestinal lumen. They sample antigens and transcytose them to APCs present in a pocket in the M cell on its basolateral side for presentation to B and T cells. Reprinted from [66] *with permission from Elsevier.*

In contrast to the absorptive villus epithelium of the intestine, the FAE contains few or no mucus secreting goblet cells and there is little mucus secretion by the FAE [67]. Dense regular microvilli and a thick glycocalyx on the apical surface of enterocytes inhibits close contact with bacteria and viruses [66,68]. The apical surface of M cells, however, differs from that of enterocytes (Figure 11): M cells have short irregular microvilli, a thin glycocalyx (20-450 nm in the rabbit PP, compared to a fairly uniform 500 nm for enterocytes) and lack integral membrane hydrolytic enzymes [67,68]. This facilitates uptake of macromolecules, organisms and particles by M cells [66,67].

M cell-mediated antigen uptake is associated with the development of a secretory IgA (sIgA) response. Polymeric IgA is transcytosed across the mucosal epithelium by

interaction with pIgR and secreted into the lumen by cleavage of pIgR forming sIgA [58]. sIgA is the dominant antibody of the mucosal immune system and provides a first line of defence in the intestine. B cells activated in PPs or the mesenteric lymph node migrate to the *lamina propria* and differentiate into IgA secreting plasma cells. IgA differs from IgG in that it functions in an external environment where the systemic mechanisms employed by IgG (such as the complement system and phagocytes) are not available. IgA therefore mostly functions as neutralising antibody providing immune exclusion of pathogens by binding to them in the lumen of the intestine and preventing epithelial adhesion and invasion [69,70]. This is highly significant, as it provides protection against the pathogen before it enters the body thus preventing infection. IgA thereby reduces the virulence of the pathogen and hence reduces the spread of infection. Mucosal vaccines may consequently be more effective at inducing herd immunity than parenteral vaccines even if their efficacy is lower [71]. IgA is well suited to function in the protease containing intestine due to its extraordinary stability [69]. As a consequence, intestinal sIgA in its native form is expelled with the faeces and is regularly measured (e.g. with ELISA) to evaluate the mucosal immune response to experimental vaccines [72–74].

2.4. Bibliography

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3. Vaccination

Vaccines function by mimicking exposure to a pathogen without eliciting the illness caused by natural infection with the pathogen. This primes an immune response to the pathogen generating immune memory against subsequent infection from organisms carrying the same antigens. Most vaccines employ a prime-boost strategy in which the vaccine is given at several different time-points referred to as the prime and following boosters. The prime generates a slow primary immune response in which memory cells are generated. The subsequent boosters activate these memory cells in fast and increasingly stronger secondary immune responses. If the vaccinated subject is later infected with the natural pathogen, this infection will correspond to reinfection with a pathogen that the immune system already knows is hostile and knows how to deal with effectively [1]. The pathogen will therefore quickly be met by a strong and specific immune response thus preventing a noticeable disease course from the infection.

3.1. Vaccine types of the present and future

Vaccines have traditionally been based on live attenuated or killed/inactivated organisms [2]. Live attenuated vaccines consist of either a weakened form of the pathogen or of a closely related but non-pathogenic organism carrying the same antigenic epitopes (e.g. Jenner's cowpox vaccine against smallpox in Chapter 1). These vaccines are the most potent and are capable of eliciting both strong humoral and cellular immune responses [1,3]. They generally require fewer administrations than inactivated or subunit vaccines, often confer lifelong immunity and can be delivered by the same route as the natural pathogen. These advantages can largely be ascribed to the close resemblance with natural infections due to their ability to replicate in the host and the presence of microbial compounds such as PAMPs that activate the innate immune system. The ability to replicate is at the same time, however, also the main disadvantage of live vaccines. In immunocompromised individuals, live vaccines may cause persistent infection and otherwise non-pathogenic strains may induce a disease course. There is also the risk that the attenuated organism reverts to virulence through random mutation and horizontal gene transfer thus regaining the ability to cause disease and possibly spread. Other drawbacks include the risk of severe adverse side reactions (e.g. local inflammation and systemic

disease), the need to handle the vaccine in a manner to keep the organism alive, and the inability of many diagnostic tests to discern between vaccinated and infected individuals [1]. Many microorganisms are in addition difficult to grow *in vitro* thus making production of whole organism vaccines impractical [4].

Inactivated vaccines consist of the disease causing agent killed or inactivated through treatment with heat or chemicals. This eliminates the ability of the vaccine to replicate thus improving safety but requiring several booster doses. Inactivated vaccines are additionally often cheaper to make and less sensitive to challenges (e.g. temperature fluctuations) during handling and storage. However, they are less potent than attenuated vaccines, often induce only weak cellular immune responses and are still associated with adverse side effects [1,2].

Advances in biotechnology have allowed more sophisticated approaches to vaccination to solve the issues associated with whole organism vaccines [5]. These include the use of DNA vaccines and subunit vaccines [6]. DNA vaccines consist of antigen encoding DNA inserted into a vector. Naked DNA in plasmid form can be coupled to nanoparticles, but a more promising method is to use recombinant vectors in which the ability of certain viruses to deliver DNA to human cells is exploited. The inserted DNA then leads to antigen production and secretion by cells in the host which can induce a strong immune response [7,8]. The existence of gene encoding DNA, however, is inherently connected to the safety concern of reversion to the disease causing virulent state [9]. An example of viral vectors is adenoviruses, which have been studied extensively for vaccines as reviewed elsewhere [10,11]. Adenovirus vectors were initially developed for gene therapy to replace missing or faulty genes, but this was abandoned due to their high immunogenicity leading to great interest in them as vaccine platform [12]. However, pre-existing or *de novo* immunity to the vector caused by natural infection prior to vaccination results in neutralising antibodies that reduce the uptake of the vaccine by APCs [10,12,13]. In an adenovirus based HIV-1 clinical trial, safety concerns lead to the termination of the study before completion of enrolment. The concern was due to elevated HIV-1 infection rates amongst vaccine recipients with pre-existing neutralising antibodies towards the adenovirus vector [14]. However, this observation was later challenged by observations from other clinical trials [15]. The issue of neutralising antibodies to the vector may be circumvented by using vectors that do not naturally infect humans [16], hiding the vectors from neutralising

antibodies through surface modification [17] and using a heterologous prime-boost approach with different vectors for prime and boosters [10].

Subunit vaccines contain one or more highly purified antigens often in the form of proteins or peptides [5]. Protein and peptide antigens present a promising direction for vaccine design as they are recognised as offering the best safety profile [18]. There is no risk of reversion to a virulent form and the toxicity associated with whole cell vaccines and inactivated toxins is removed. Subunit vaccines also offer advantages in terms of production and licensing since consistent production of well-defined protein/peptide antigens can be done relatively easily and inexpensively [19]. Furthermore, subunit protein antigens from different pathogens can be combined in fusion proteins to create a vaccine against more than one infection [20]. The main disadvantage of subunit vaccines is a low immunogenicity. The high purity of the antigenic fragments means that microbial danger signals are not present and thus, the antigen is often not recognized as hostile by the immune system. This often results in the antigen being ignored by the immune system and may result in tolerance [19,21]. In order to achieve effective immunisation with subunit vaccines, the antigen must therefore be delivered with adjuvants (section 3.3). Appropriate choice of adjuvant allows stimulation of immune responses pertinent to the pathogen against which the vaccine is designed. Adjuvanted subunit vaccines can stimulate strong cellular immune responses in addition to humoral immune responses and are thus promising for vaccination against targets that have proven difficult to vaccinate against with inactivated vaccines [19].

3.2. Oral vaccines

Oral vaccines against four enteric pathogens are routinely administered to humans. Examples of licensed vaccines are summarised in Table 1. Another oral vaccine against adenovirus Type 4 and Type 7, an upper respiratory tract disease, is currently approved by the Food and Drug Administration of the United States of America to military personnel. Oral delivery of the virus results in asymptomatic infection of the GI-tract leading to immunity in the upper respiratory tract. All of the oral vaccines exist as live attenuated vaccines based on the enteric pathogen that naturally has adapted to evade and cross the biological barriers of the GI-tract (section 2.2). The cholera vaccine, however, exists also in the form of inactivated vaccines, e.g. in the form of a recombinant cholera toxin B

subunit and whole cell inactivated *vibrio cholera* O1 vaccine proving that oral vaccination is possible with non-live vaccines [11,22].

Table 1. Licensed oral vaccines compiled from [11,22] *(non-exhaustive list).*

Trade name	Infection	Vaccine
Sabin	Poliomyelitis	Live trivalent attenuated polio vaccine, Sabin strains 1,2,3
Dukoral	Cholera	Cholera toxin B subunit and inactivated whole cell *Vibrio cholera* 01
Vaxchora	Cholera	Live attenuated cholera vaccine, CVD-10-HgR
Vivotif	Typhoid	Live attenuated strain, Ty21a
RotaRix	Rotavirus	Live monovalent attenuated human rotavirus, RIX4414 strain of G1P(8) type
RotaTeq	Rotavirus	Live pentavalent attenuated rotavirus reassortants derived from human and bovine species
None	Acute respiratory disease	Live adenovirus Type 4 and 7

Live viral vectors (section 3.1) have been widely investigated for oral vaccination and shown to be able to stimulate potent immune responses after oral administration [9,11]. However, subunit vaccines are desirable since they have better safety and their production is not hampered by difficulties in culturing microorganisms [9]. Nevertheless, oral administration of subunit vaccines is challenging. Protein antigens do not have the natural ability of enteric live attenuated vaccines to survive the chemical and enzymatic attack in the GI-tract and cross the biological barriers (section 2.2). The residence time in the small intestine is limited (3-4 h in humans [23] and much less in mice (Paper III, Chapter 8) demanding fast presentation of the antigen to APCs. Furthermore, not all adjuvants are active at mucosal surfaces. Oral subunit vaccines therefore need effective delivery systems. Several strategies to deliver protein antigens orally have been reported [9,11,22], some of which will be presented in section 3.3.2, but at present, none have been licensed for human use.

3.3. Adjuvants

Adjuvants (from *adjuvare* – to help) are substances delivered together with an antigen to improve and modulate the immune response to the antigen [24]. The concept was first described by Ramon in 1925 and since then, a large group of structurally heterogeneous adjuvants have been developed [25]. Today, it is generally accepted that subunit vaccines need to be co-formulated with adjuvants to be able to generate effective immunisation against the antigen. The wide range of adjuvants stimulate immune responses in many different ways, however, they can be divided into immunopotentiating compounds (often referred to simply as adjuvants) and delivery systems. An immunopotentiating compound is a substance that stimulates and/or modulates the immune response to an antigen while delivery systems help to deliver the antigen effectively to APCs. In practice, delivery systems such as particles systems often have intrinsic immunostimulatory effects. However, the combination of antigen with a particle system and immunopotentiator(s) is a common strategy to achieve a strong immune response to the subunit antigen [24,25].

3.3.1. Immunopotentiators

A wide range of immunopotentiating compounds have been studied as reviewed in several places [9,26,27]. These include aluminium salts, bacterial components such as PAMPs, synthetic bacterial DNA, double stranded viral RNA, saponins, cytokines, a range of synthetic small molecules and other substances. However, only few have been used in licensed human vaccines [28].

3.3.1.1. Aluminium salts

Aluminium salts are the most widely used class of adjuvants and include aluminium phosphate, aluminium hydroxide and aluminium hydroxyphosphate [29,30]. The class was first discovered in 1926 [31] and introduced as adjuvant for inactivated diphtheria and tetanus toxoids in 1932. Since then, aluminium salts remained the only adjuvant in licensed human vaccines for approximately 70 years [28,32]. Aluminium salts have been used either by adding antigen to aluminium salt solution to form antigen-aluminium salt precipitate or by adding antigen solution to a preformed aluminium gel such that the antigen is adsorbed to the gel [33]. The latter has generally been preferred since it allows for much more

controlled and homogenous aluminium formulations because the antigen does not affect the structure of the adjuvant [33,34]. Both types of aluminium-based adjuvants are generally (although inaccurately as explained by Gupta [33]) referred to simply as alum [33] and this custom will be continued here.

The mechanism of the adjuvant effect of alum is only recently beginning to be understood. Alum was long believed to function through a depot effect allowing the antigen to remain at the injection site for extended periods of time. This has since been disproven and a range of other mechanisms have been indicated. These include stimulation of the innate immune system through inflammation and enhanced antigen uptake by APCs through the particulate nature of alum. However, the molecular mechanisms are still debated [29,30,32].

The key advantage of alum is that it has been used for almost a century and has a proven safety record [30,31]. Alum acts primarily by increasing antibody production and is therefore mostly effective against pathogens that can be eliminated by antibodies such as extracellular bacteria [28]. However, alum is often less potent than other adjuvants and cellular immune responses to alum-adjuvanted vaccines are almost absent. Alum-adjuvanted vaccines have consequently not been effective against intracellular pathogens and other targets that cannot be eliminated through antibodies alone such as HIV, tuberculosis and malaria [28,34]. Other drawbacks include unpredictable adsorption of certain proteins and low stability of some adsorbates [35]. The immunogenicity of alum depends on the adsorption of antigen to alum [29,33]. This adsorption depends on physical and chemical characteristics of the antigen, the type of alum and conditions of adsorption (e.g. pH, temperature, size of gel particles and ionic strength of the mixture) and alum has therefore been described as difficult to manufacture consistently leading to batch-to-batch variations [33]. There are also some safety concerns related to the use of alum, such as the stimulation of IgE antibodies which may lead to IgE mediated allergic reactions [33]. Nonetheless, alum remains the gold standard for vaccination [35].

3.3.1.2. Bacterial toxins

Alum is a poor inducer of mucosal immunity [27,36] and is not suitable for oral delivery [1]. Other adjuvants are therefore necessary for oral vaccines. The best studied mucosal adjuvants are the bacterial enterotoxins cholera toxin and *Escherichia coli* enterotoxin and

mutants and subunits of these [36]. These adjuvants increase the epithelium permeability and modulate the vaccine uptake by APCs and the response of lymphocytes. They have been used successfully for oral immunisation of mice, but are too toxic for use in humans [9,22].

3.3.1.3. Saponins

The adjuvant activity of plant saponins has been known since 1951. In 1970, Dalsgaard showed a correlation between the source of the saponin and its activity. The most active saponins were those obtained by aqueous extraction from the cortex of the South American tree *Quillaja saponaria* Molina, also known as Soapbark tree or Panama wood [37,38]. Further purification to remove tannins and polyphenols results in a mixture of saponins known as Quil-A [38]. Quil-A consists of a mixture of triterpenoid- or steroidal glycosides [39]. Quil-A saponins are thus surfactant molecules with a hydrophilic (sugar) – lipophilic (aglycone) – hydrophilic (sugar) structure [38] and have been reported to form micelles in water at concentrations above 0.03 % [40].

The use of Quil-A in humans has been limited by reports of toxicity and by the difference in glycoside composition between different batches stimulating interest in purifying and characterising specific fractions [41,42]. Quil-A has been separated into 23 HPLC fractions comprising more than 60 individual compounds [42]. Some fractions of Quil-A retain the immunogenicity while showing improved safety over Quil-A [41,43]. One of the major fractions termed QS-21 is known for low toxicity and is the only fraction to have been evaluated in humans [44]. The chemical structure of QS-21 is shown in Figure 12. However, the use of QS-21 is associated with a number of limitations: difficulty in the purification process of QS-21 leads to yields lower than 0.0001 %, toxicity limits the dose to about 50 µg in human vaccines and QS-21 is unstable in aqueous solution at physiological pH at room temperature. These limitations for QS-21 has led to the development of safer and more stable synthetic variations suitable for large scale manufacturing [44]. However, Quil-A is heavily used in veterinary vaccines [45].

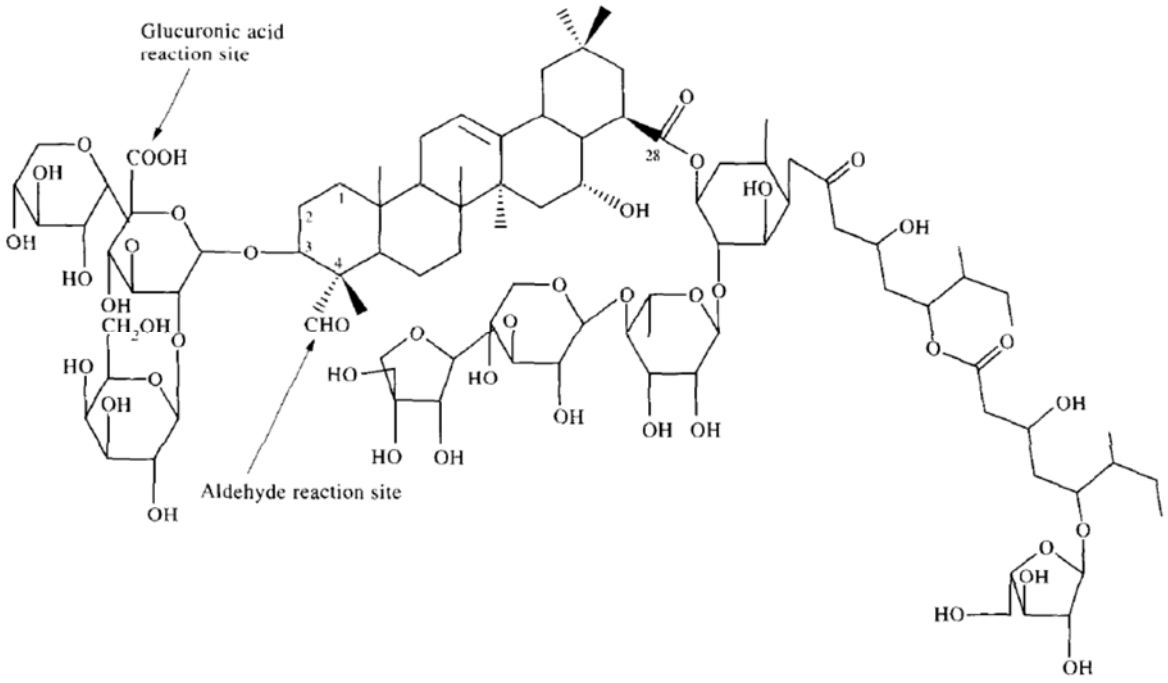

Figure 12. Chemical structure of QS-21. QS-21 is a purified fraction of the adjuvant Quil-A, which comprises a range of related structures. Reprinted from [46] *with permission.*

Quil-A has been studied in formulations with liposomes [47–51], immune stimulating complexes [52–57] and cubosomes [47,58]. It is a TLR-independent adjuvant capable of stimulating strong humoral and cellular immune responses (Paper I, Chapter 6). One of the particularly interesting characteristics of Quil-A, is its ability to stimulate a strong $CD8^+$ CTL response, which is often difficult to obtain with non-replicating vaccines. CTL responses are crucial for protection against intracellular infections by killing infected or transformed (e.g. cancer) cells [38,59]. Saponins interact with cholesterol in lipid membranes giving Quil-A the ability to create pores in cell membranes [60,61]. This has been proposed to be an important mechanism for the immune activation of Quil-A as it may allow the vaccine to enter the cytosol of APCs (likely through endosomal escape). Quil-A thereby facilitates cross-presentation of the antigen and hence the stimulation of CTL responses. However, the mechanism of action of Quil-A is still not fully understood and other activities have been reported. These include inflammasome activation and co-stimulation of T cells through binding to T cell receptors [62,63].

Saponins are well tolerated when administered orally and crude forms of quillaja saponins are used as foaming agents in many food, beverage and cosmetic products [39,64].

Saponins form a mucosally active adjuvant capable of stimulating humoral and cellular immune responses when administered orally [39,65,66]. The surfactant property of Quil-A means that it can act as a penetration enhancer. In a study on transcutaneous vaccines, Rattanapak et al. reported that cubosomes (described in section 3.3.3.3) increased the skin permeation of a peptide antigen and that the addition of Quil-A significantly further enhanced this effect [67]. The interpretation of this was that Quil-A increases skin penetration of peptides. It is possible that a similar effect may be seen at other non-invasive routes.

3.3.1.4. Dextrans

Dextran is a class of high molecular weight (MW) branched polymers composed of D-glucose units. It is produced by a variety of bacteria and its physicochemical properties depend mainly on their MW and degree of branching which is determined by the bacterial source. Dextrans have been used in a range of drug delivery applications e.g. as oral tablet excipient, drug stabiliser and as controlled release polymer excipients.

Dextran is mucoadhesive and can act as mucus permeation enhancer in mucosal protein delivery [9]. It has also been used as adjuvant in combination with other adjuvants, in a chemically modified form to improve its immunogenicity and in a cross-linked form creating microparticles [1]. Dextran microparticles have been associated with strong humoral immune responses when given together with alum and can substitute substantial amounts of alum thus reducing toxicity [68].

3.3.2. Particulate delivery systems

The natural pathogens that the immune system has evolved to respond to are commonly of particulate nature. A wide range of particulates have been developed with sizes similar to those of natural pathogens as illustrated with classes of particles in Figure 13 [69]. Antigen delivery to APCs with particle formulations has been shown to be 1,000-10,000 times more efficient than delivery of soluble antigen [70]. As mentioned in section 3.3, the particle formulations are often also combined with immunopotentiators that can strongly increase the immunogenicity of the particulate vaccine and may be used to polarise the immune response towards a humoral or cell mediated response [71]. Furthermore, it is known that

soluble antigens generally are inefficiently cross-presented, whereas particulate forms of antigens of similar or larger size than viruses are more readily cross-presented [69]. It is therefore not surprising that antigens incorporated in micro- and nanoparticle formulations inherently are more immunogenic than soluble antigens [21]. Several physical and chemical properties of particles can affect their ability to deliver antigen to APCs and stimulate an immune response to the antigen [21,72]. These include the size, surface charge and incorporation of antigen as will be discussed below.

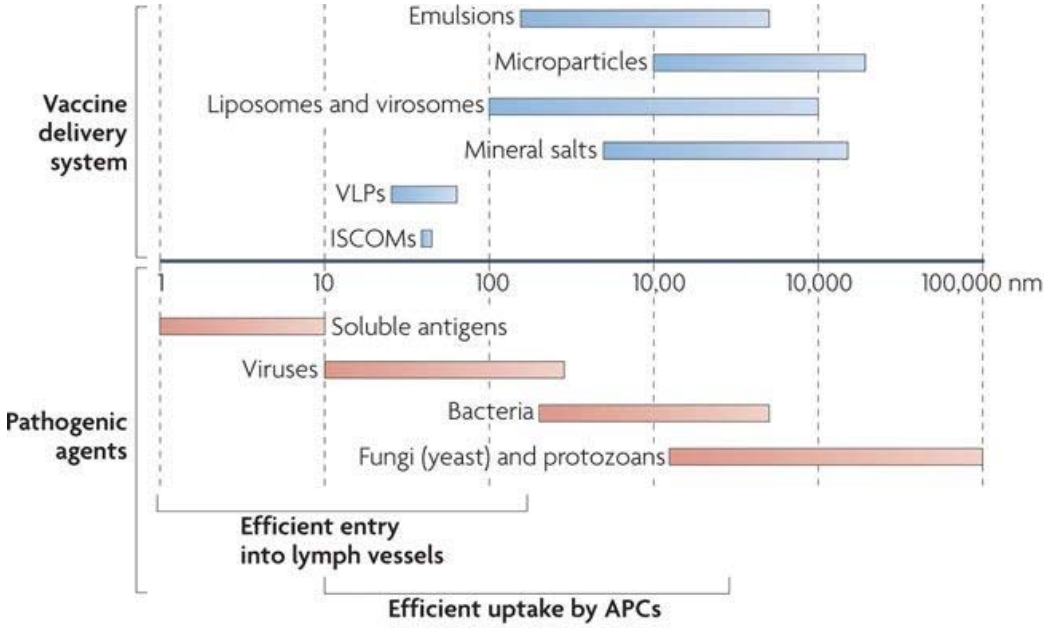

Figure 13. Sizes of delivery systems and pathogenic organisms. A range of particle delivery systems have been developed with sizes similar to those of natural pathogens such as viruses and bacteria. Antigens delivered in nano- and microparticles are delivered much more efficiently to APCs than soluble antigens. Reprinted from [69] *with permission.*

3.3.2.1. Particle size

APCs have been reported to process particulate antigens ranging in size from 8 nm to 3 µm [21,69]. There is no clear indication of an optimal size within this range as reports often are in disagreement. These conflicting studies may be related to the different injection sites, methods of evaluation and particle compositions – the latter of which is often used to control the particle size [21,73]. Particles in the size range of 20-200 nm efficiently enter the lymphatic system in contrast to particles in the size range of 200-500 nm which must be carried into the lymphatic system by APCs [69]. However, newer studies have suggested that 1 µm large particles can also enter the lymphatics [74]. Smaller particles are reported

to be cleared faster from the injection site than larger particles and it has been suggested that after subcutaneous (s.c.) administration, small particle size may bias immune responses towards humoral responses and larger particles towards cellular immune responses [21,73]. However, following intra-muscular administration of a cationic liposome formulation, small and large liposomes (~200, 600, 1500 and >2000 nm) were seen to drain similarly fast from the site of injection, but larger particles were detected in higher levels in the draining lymph node. Based on cytokine and antibody measurements, the authors concluded that the type of immune response and the antibody production was unaffected by particle size, but that medium sized particles (~600 nm) were the most efficient at stimulating expression of pro-inflammatory cytokines [75].

For oral drug delivery, mucus can present a barrier that must be overcome to allow intestinal absorption of the drug. Particles can be engineered to avoid mucoadhesion and thus be able to diffuse through the mucus if they are sufficiently small to penetrate the mesh of the gel layer [76]. Particles as large as 500 nm have been reported to be able to diffuse through the mucus if made with suitable surface characteristics [77] as discussed in section 3.3.2.2. However, the biological barrier of mucus might be less pronounced for vaccines, since M cells are covered by a thinner mucus layer than the enterocytes outside the FAE [78] and since dendritic cells in the *lamina propria* are able reach into the intestinal lumen to sample antigens (section 2.3). M cells have been reported to transport particles of many types ranging in size from 20 nm to 10 µm. A lower size limit has not been established, but the upper limit is thought to be approximately 10 µm [79]. PP uptake of particles of size 1 µm has been observed to be equally as efficient as the uptake of 0.3 µm particles [80], but particles of 1-2 µm appear to be taken up more efficiently than larger particles [79,80].

3.3.2.2. Surface charge of particles

The surface charge of particles is often evaluated by measuring the zeta potential of the particles [72,81]. The zeta potential is not necessarily equal to the surface charge, but it is often close and is readily measurable [82]. Zeta potentials have been correlated to the colloidal stability of particles [72,81] with increasing positive or negative charge resulting in increased colloidal stability through electric repulsion. Guidelines classify particles with zeta potentials of magnitude 0-10 mV and 10-20 mV to be highly unstable and relatively stable, respectively, whereas particles with zeta potentials of 20-30 mV and > 30 mV are

moderately or highly stable, respectively [83]. However, while colloidal stability depends on both electrostatic repulsive forces and van der Walls forces, zeta potentials only provide information about electrostatic repulsion [83]. Steric interactions from large molecules can also provide colloidal stabilisation and thus particles with zeta potentials much closer to zero than indicated in the guidelines above might have excellent colloidal stability [83,84].

Particles with a positive surface charge are generally taken up more efficiently by APCs than negatively charged particles. This has been attributed to electrostatic interactions between the cationic particles and negatively charged cell membranes, and has been used to increase the immunogenicity of particles administered parenterally, nasally and to the pulmonary mucosa [21,72,73]. Cationic particles are additionally mucoadhesive due to electrostatic interactions with the negatively charged mucus (section 2.2) [85]. Mucoadhesion may increase the intestinal residence time of particles [78,86] and hence may prolong the exposure of the vaccine to the inductive lymphoid tissues of the intestine. It is also possible that mucoadhesion might reduce the dilution of the vaccine by gastrointestinal secretions [85]. However, it remains unclear whether a cationic particle surface charge is advantageous for oral vaccine delivery systems [87]. Mucoadhesive particles are likely to be trapped in the mucus and thus may not efficiently cross the mucus barrier [78,86,88]. Although PPs are covered by a thinner layer of mucus than the adjacent intestinal epithelium (section 2.3), they only account for ~1 % of the total surface area of the small intestine. It is therefore possible that the mucus barrier covering the normal absorptive epithelium might trap and clear particles targeted to the M cells before they reach the PPs [78]. Mucoadhesion could exacerbate this effect and thus other strategies might be preferable for oral vaccination. Hydrophilic and neutral or slightly negatively charged particles have been shown to be optimal for mucus penetration [89]. Such particles may further be produced to shed an outer coating and expose an inner cationic particle once they reach the epithelium for efficient uptake by APCs [88]. Other studies have found that highly negatively charged particles provide optimal particle uptake by PPs *in vivo* [80,90]. In summary, while cationic particles are generally accepted to increase the immunogenicity of parenterally administered vaccines, it is controversial how a cationic surface charge might affect the oral immunogenicity of vaccines. These controversies may in part be due to the effect of the composition of the particles used in the experiments. The hydrophobicity of the particle surface, for example, is thought to be an important factor for mucoadhesion [78,91]. Mucus is also known to cause agglomeration resulting in increased effective

particle size and increased trapping [91]. In addition, it is important to remember that zeta potentials are heavily dependent on the pH and also depend on the electrolytes and the particle concentration in the aqueous medium [83]. Perrie et al. measured zeta potentials of a cationic liposome formulation to be ~15 mV in simulated intestinal fluid (pH 7.4) and ~40 mV in simulated gastric fluid (pH 2) [92]. The same group later measured the effect of varying pH on the size and zeta potential of anionic lipid vesicles as they have summarised in a figure [93] reprinted in Figure 14. Furthermore, nanoparticles are known to be coated with a protein corona *in vivo* which can change their size and zeta potential [94,95].

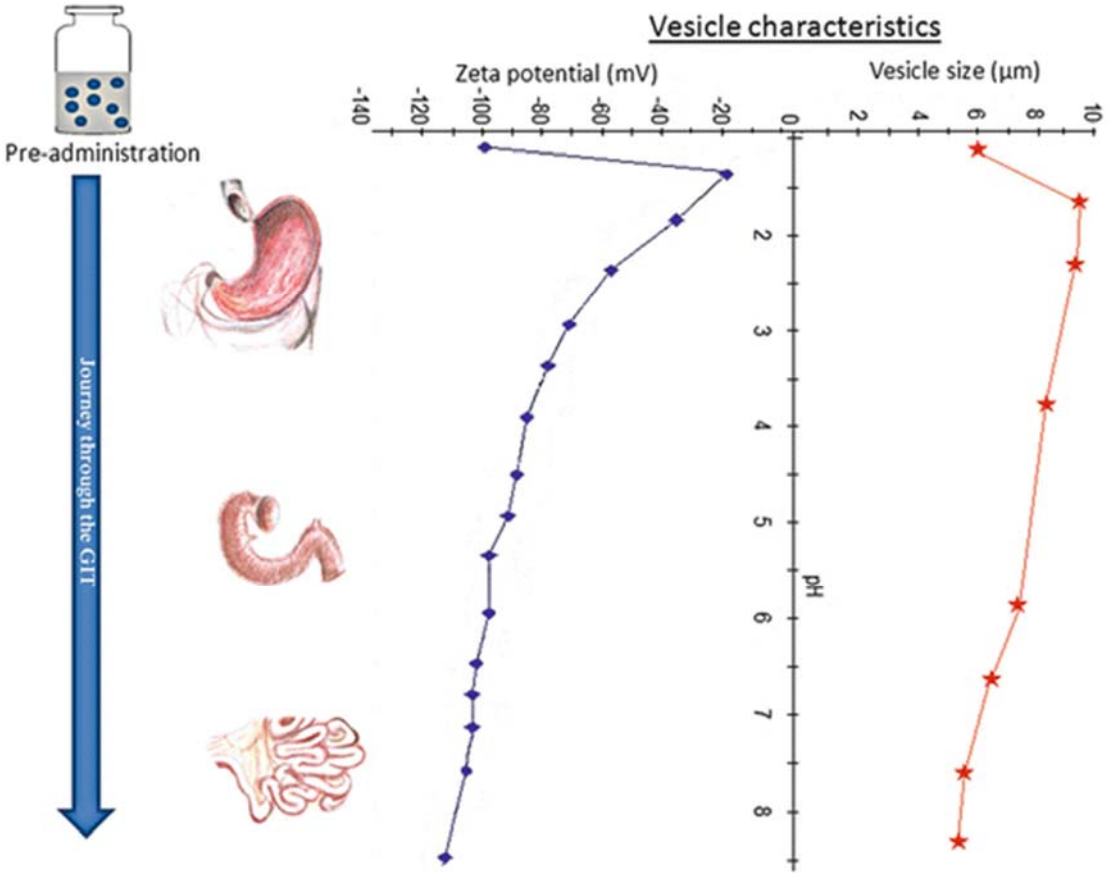

Figure 14. Effect of pH variation in the human GI-tract on the size and zeta potential of a liposome formulation. Both the size and the zeta potential are heavily affected by the pH of the buffer.

3.3.2.3. Co-delivery of antigen and adjuvant

It has been an area of controversy in the literature whether physical linkage of antigen and adjuvant is important for the immunogenicity of a vaccine. Kamath et al. reviewed a range of reports showing that physical linkage of antigen to immunopotentiator, either directly by conjugation or within a formulation, is required to stimulate strong immune responses

[96]. In contrast to this opinion, Coffman et al. stated in an earlier review that antigens do not need to be incorporated into a particle, but may be administered as soluble antigen together with antigen-free particles [71]. Indeed, White et al. reported that liposomes with mannosylated lipid core peptide (manLCP) constructs stimulated similar levels of antigen specific cytotoxicity when formulated in liposomes with Quil-A as when it was simply admixed with liposomes with Quil-A [49]. However, they also observed that when manLCP and Quil-A were formulated in separate liposomes, the immune response was reduced to a level comparable to manLCP liposomes without Quil-A. They therefore speculated whether soluble manLCP might adsorp to Quil-A containing liposomes. This would effectively create liposomes with both Quil-A and manLCP and thus support the notion that adjuvant and antigen need to be delivered in the same particle [49]. Kamath et al. similarly observed (using a different vaccine system) that administration of antigen and adjuvant in two separate syringes at the same site did not prevent the combined antigen/adjuvant activation of DCs [96]. Inspired by this, they investigated the kinetic determinants of adjuvanticity by varying the kinetics with which adjuvant and antigen were delivered to the draining lymph node. They concluded that the crucial element for stimulating a strong cellular immune response is to avoid exposure of lymph node resident DCs to antigen before activation by the adjuvant. Physical linkage was not necessary, but it was important that the adjuvant was delivered to the individual DCs at the same time as the antigen or earlier. The reason for this was suggested to be that antigen presentation without activation signals might result in tolerance rather than immune stimulation. Synchronous delivery of antigen and adjuvant was, however, not a requirement for antibody responses [96].

3.3.3. State of the art particle systems

A selection of common particle systems used in experimental vaccines is illustrated in Figure 15 [9,11,97–99].

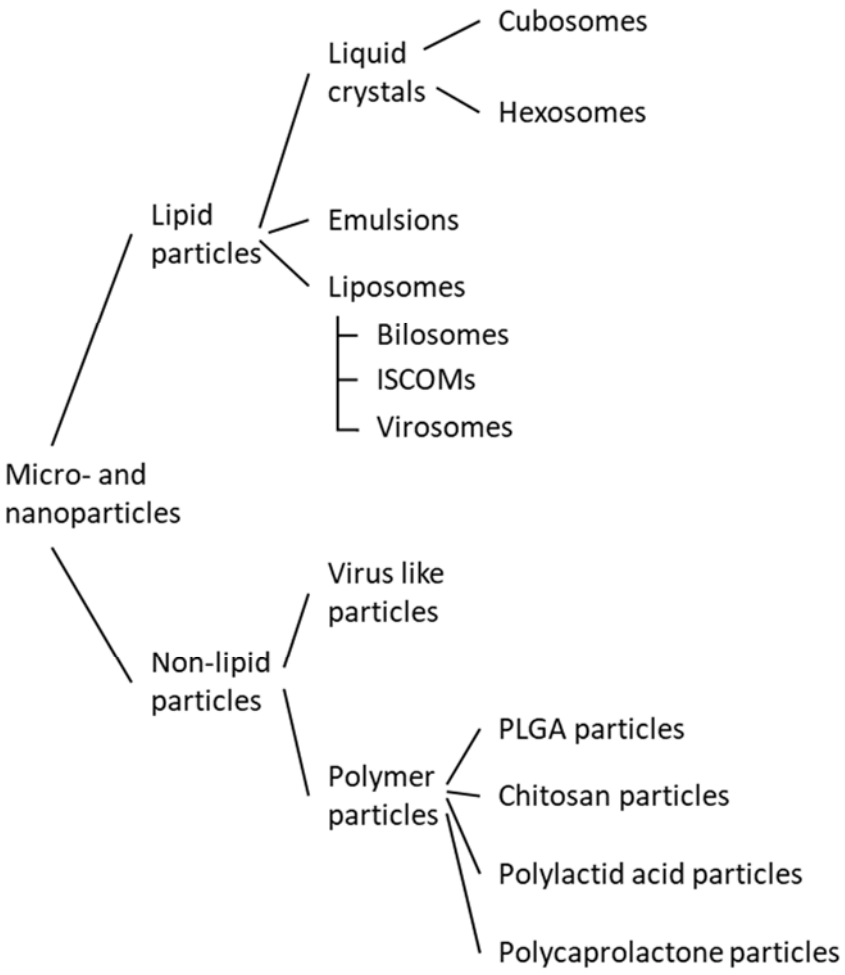

Figure 15. Selection of particle formulation classes and related structures used for vaccine delivery in experimental systems [9,11,97–99].

3.3.3.1. Lipid-based particles

A wide range of lipid particles have been developed including emulsions, liposomes, liposome-related structures and liquid crystals. A variety of emulsion-based adjuvants have been produced and some have been used in human and veterinary vaccines [98,100]. Liposomes and the related bilosomes and ISCOMs are discussed in the following, whereas lipid-based liquid crystals shall be discussed in section 3.3.3.3 and further in Chapter 4.

Liposomes were first discovered in 1965 by Bangham et al. [101]. The use of liposomes as adjuvants for entrapped diphtheria toxoid antigen was reported by Allison and Gregoriadis in 1974 [102] and liposomes have since then been studied extensively as vaccine adjuvants [73]. Liposomes are spherical particles with one or more lipid bilayers surrounding an aqueous core (Figure 16). The lipid bilayer is often made from amphiphilic phospholipids

and cholesterol [19]. However, other lipids can also be used and it is possible to control the physicochemical properties of the liposome by varying the composition and manufacturing method [73]. The amphiphilic nature of the lipids facilitate self-assembly in water forming bilayers with the hydrophobic ends facing each other and the hydrophilic ends facing the aqueous environment [103]. This structure allows antigens and adjuvants with different properties to be incorporated into the same liposome. Hydrophobic substances are often incorporated directly into the lipid bilayer and hydrophilic substances may be encapsulated in the centre of the liposome or bound to the liposomal membrane [73]. Binding to the membrane can be through adsorption, electrostatic complexation with oppositely charged lipids or by conjugation to an amphiphilic anchor inserted into the lipid membrane (Figure 16). The optimal loading strategy depends on the function of the compound and on the physicochemical properties of the liposome, antigen and adjuvant [73].

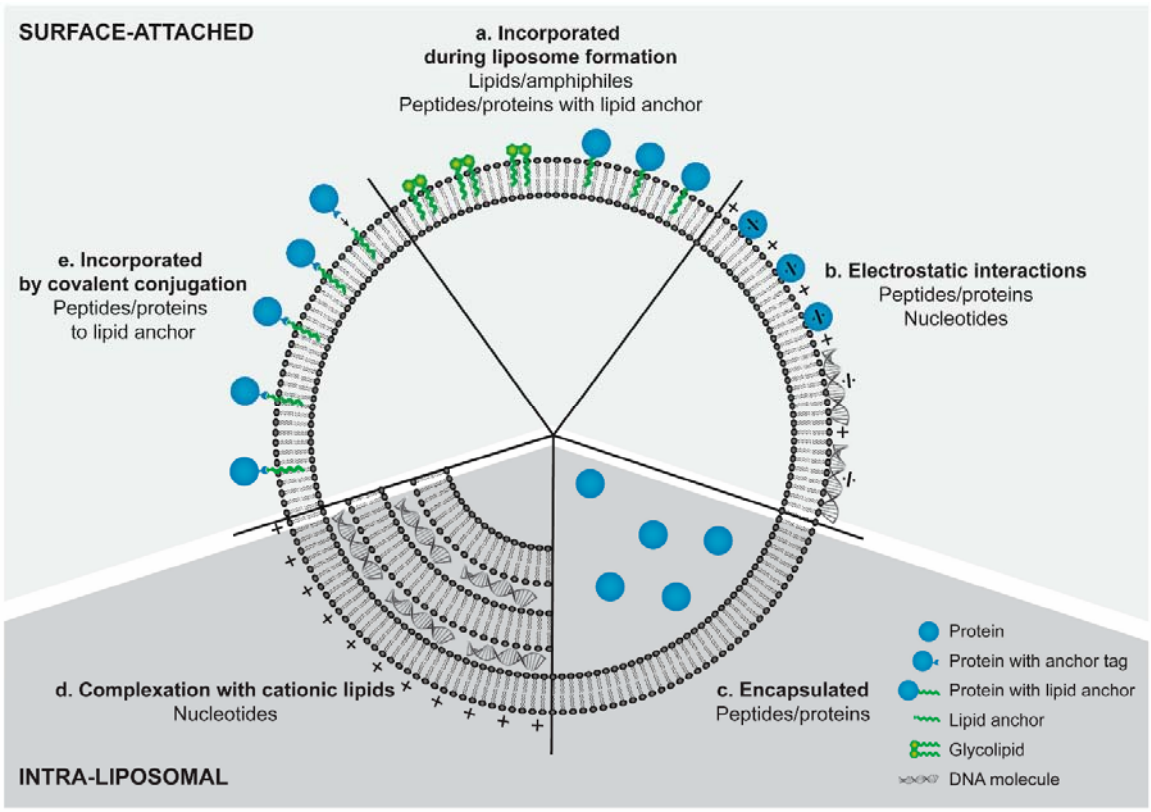

Figure 16. Schematic of the liposome structure and different loading strategies. Liposomes are composed of one or more lipid bilayers and an aqueous lumen. Several strategies exist for loading of hydrophilic and hydrophobic actives. a) lipophilic and amphiphilic actives can be incorporated into the lipid membrane, b) charged hydrophilic actives can be attached to an oppositely charged bilayer membrane by electrostatic interactions, c) hydrophilic actives can be encapsulated in the centre of the liposomes, d) nucleotides can be complexed with cationic lipids to become embedded between multiple lipid bilayers and e) hydrophilic actives can be covalently conjugated to lipid anchors incorporated into the lipid bilayer. Reprinted from [73].

Some adjuvants elicit unwanted side effects when administered freely. For example, the TLR-4 agonist monophosphoryl lipid A (MPLA) (the active moiety of a bacterial endotoxin lipopolysaccharide [104]) is pyrogenic [73] and saponins have haemolytic effects [43]. These side effects can be reduced by incorporation of the adjuvants into particles such as liposomes [41,73].

Large development efforts have led to an array of vaccines with liposome based adjuvants being tested in clinical trials [73]. Two liposome-based adjuvants, virosomes and Adjuvant system 01 (AS01), have been used in vaccines licensed for human use or approved for pilot routine administration in selected countries where there is a high risk of infection, as reviewed elsewhere [105]. Virosomes (first licensed in a vaccine for human use in 1994)

are viral envelopes from influenza virus that have been emptied for inner core and genetic information, thus essentially becoming liposomes with viral envelope proteins on the surface. AS01 is a liposome adjuvant containing the immunopotentiators MPLA and QS-21 [105].

Liposomes are prone to membrane disruption by bile salts. Bilosomes are based on vesicles (niosomes) similar to liposomes formulated with bile salts in the lipid bilayer. The presence of bile salts in the lipid membrane protects it from emulsification by endogenous bile salts in the intestine. Bilosomes can thus protect encapsulated antigen from the harsh environment of the GI-tract and are able to stimulate humoral and cellular immune responses following oral administration [11,19].

Immune stimulating complexes (ISCOMs) are another derivative of liposomes composed of phospholipids, cholesterol and saponins (often Quil-A) [11]. ISCOMs are self-assembled spherical hollow nanoparticles with a characteristic open cage-like structure similar to the stitching on a football and are often around 40 nm in diameter [11,106]. Quil-A is known to interact with cholesterol in lipid membranes (section 3.3.1.3) and this interaction is likely the reason for the unique structure of ISCOMs. Indeed, in the ternary phase diagram of phosphatidyl choline (phospholipid), cholesterol and Quil-A, ISCOMs form only when all three components are present [107]. The incorporation of Quil-A into ISCOMs reduces the toxicity of Quil-A while retaining its adjuvant effect [98].

ISCOMs have been shown in several animal models to be potent adjuvants stimulating a balanced humoral and cellular immune response [11,39]. They have also shown promise as oral vaccine system in a range of studies, as reviewed by Sjölander and Cox [39]. However, while oral administration of some antigens in ISCOMs could stimulate immune responses, no effect was seen towards other antigens despite those vaccines being effective after parenteral administration. Another vaccine was ineffective after three oral administrations, but stimulated a serum IgG response and IgA secretion in the mesenteric lymph node and PPs following intraperitoneal priming and oral boosting. The reasons for the efficacy differences are unknown, but may be related to the antigens, differences in the ISCOM formulations, the doses of antigen and Quil-A as well as the employed immunisation schedules [39].

3.3.3.2. Non-lipid based particles

Virus like particles (VLPs) are self-assembled nanoparticles in the size range of 20-200 nm composed of viral capsid proteins. They can resemble the authentic geometry of the virus they are sourced from, but do not contain any of its genetic material and are thus a safer alternative to attenuated virus [21,108]. VLPs have been shown to be potent adjuvants for stimulating CTL responses. Fusogenic properties allow them to disrupt or fuse with cell membranes and deliver their antigen cargo directly into the cytosol of APCs thus facilitating cross-presentation of the antigen [109]. The viral proteins comprising the shell of the particle forms repetitive arrays of conformational epitopes. These function as immunopotentiators through cross-linking of B cell receptors and thus stimulate potent antibody responses [24,69]. The particulate and repetitive structure further causes effective opsonisation and uptake of VLPs by APCs [108]. VLPs are consequently highly immunogenic without additional immunopotentiators, but can be even more potent together with immunopotentiators [69]. VLPs made from an orally transmissible virus have furthermore shown promising results for the use of VLPs for oral vaccination [110].

An important property of any vaccine that should not be overlooked is the simplicity and ease of manufacture [19]. Since VLPs spontaneously self-assemble from their protein components, the main manufacturing challenge is production and purification of those proteins. VLP proteins can be produced in expression hosts such as bacteria, insects, yeast and mammalian- or plant cells. Yeast cells in particular allow for rapid and efficient production of large quantities of VLP proteins and are used for production of commercial VLP based vaccines [108]. VLPs were the first nanoparticles used with a subunit antigen vaccine to be licensed in the United States of America in 1986. As of 2016, four VLP-based vaccines are licensed for human use and more were under preclinical and clinical development [21].

Poly(lactic-co-glycolic) acid (PLGA) is a biodegradable and biocompatible synthetic polymer. PLGA has been studied extensively for a range of biomedical applications leading to great knowledge of the characteristics of the polymer formulation and therefore how to achieve desired particle properties such as degradation rate, release kinetics, size, and specific surface characteristics through coating with other polymers. Furthermore, the existing use of PLGA particles in parenteral drug formulations licensed for human use might expedite approval of new PLGA particle-based products [111]. The main advantages

of PLGA particles for vaccine purposes are their high loading capacity, their ability to protect encapsulated antigen from harsh *in vivo* conditions, and the possibility for sustained concurrent release of antigen and immunopotentiator [112].

PLGA particles have shown promise for single-injection systems of both subunit vaccines (reviewed by McHugh et al. [113]) and the inactivated polio vaccine [114]. However, they may not be an optimal system for immunisation in prime-boost regimens. Kirby et al. reported that PLGA particles stimulated weaker immune responses than similarly adjuvanted liposomes after s.c. administration [115]. Furthermore, PLGA particles encapsulating antigens are associated with antigen stability issues due to exposure to organic solvents during formulation and acidification of the microenvironment inside the particles during polymer degradation [113]. Inclusion of basic excipients to neutralise this acidification has been reported to be crucial for the immune response to some vaccine antigens [116].

Oral vaccination using PLGA particles has been attempted with some success. PLGA particles with the immunopotentiator MPLA and ovalbumin (OVA) as antigen were able to stimulate a weak humoral immune response after oral administration similar to that of s.c. injected soluble OVA [111]. In a newer study, PLGA nanoparticles carrying an HIV antigen and three TLR ligand immunopotentiators were encapsulated in microparticles consisting of the pH-sensitive polymer Eudragit® FS30D (soluble at pH > 7) for large intestinal delivery. Oral administration of this vaccine formulation resulted in some protection against rectal viral challenge showing promise for the use of PLGA nanoparticles for oral vaccination when used together with enteric coatings [117].

3.3.3.3. Liquid crystal nanoparticles

Certain amphiphilic lipids can self-assemble to form liquid crystal structures in excess water. Lyotropic liquid crystalline phases include the lamellar, cubic and hexagonal phase [118]. A further discussion of these liquid crystal phases is provided in Chapter 4. Liquid crystalline phases can be macroscopic and, if prepared from amphiphiles with very low aqueous solubility, dispersed to form nanoparticles with the same internal structure as the bulk phase. Particles formed from the lamellar liquid crystalline phase are known as liposomes and were discussed in section 3.3.3.1. Particles based on the inverse hexagonal

phase are referred to as hexosomes and those of the inverse cubic phase as cubosomes [118]. Non-lamellar liquid crystalline structures such as cubosomes and hexosomes show fusogenic properties which have been suggested to contribute to delivery of antigens directly into the cytosol of APCs thus facilitating cross-presentation for stimulation of a CTL response [119].

Cubosomes consist of a highly contorted lipid bilayer forming a continuous lipid domain, inside which is a network of two tortuous but non-intersecting water channels. Compared to liposomes, cubosomes have greater membrane resistance to rupture and their structure gives a much larger surface area relative to the particle volume [112]. The structure of cubosomes with hydrophilic and hydrophobic domains provides a similar ability to that of liposomes to incorporate compounds of different physicochemical properties. Cubosomes are as a result flexible in the types of antigens and adjuvant they can incorporate. However, since cubosomes have much more membrane than similarly sized liposomes, they may offer increased incorporation of active compounds over liposomes [120]. The use of cubosomes for vaccine purposes is relatively new. Rizwan et al. showed that cubosomes with MPLA and imiquimod as adjuvants, and OVA as antigen stimulated a strong immune response [120]. When compared to similarly adjuvanted liposomes and to OVA with alum, cubosomes stimulated equally strong humoral immune responses and significantly stronger cellular immune responses [120]. Cubosomes were also tested as single-injection vaccine by the same group using Quil-A as immunopotentiator [47]. Gordon et al. observed a lower antibody response of single injected cubosomes compared to OVA and alum given with a booster 14 days after the prime indicating that a booster is necessary when using cubosomes [47]. In a later study, cubosomes were used to potentiate the immune response to an inactivated virus with a polysaccharide immunopotentiator [97]. In all referenced studies, the cubosomes were made from the lipid phytantriol (presented in Chapter 4).

Hexosomes are similar in structure to cubosomes, but their lipid membrane contorts into the inverse hexagonal structure instead of the inverse cubic (described in section 4.2.2). Like cubosomes, hexosomes provide a high loading capacity and flexibility with the actives they can incorporate [99], and have only recently been evaluated as vaccine carriers. The synthetic analogue of the mycobacterial lipid monomycoloyl glycerol (MMG) known as MMG-1, is known to be a strong stimulator of cellular immune responses. Rodrigues et al. designed hexosomes based on phytantriol with MMG-1 as immunopotentiator and the

chlamydia trachomatis serovar D major outer membrane protein as antigen [99]. The hexosomes were compared to a cationic liposome formulation of similar particle size and with the same adjuvant named Cationic Adjuvant Formulation 04 (CAF04) [99]. CAF04 had been shown earlier by the same group to be a strong stimulator of humoral and cellular immune responses [121]. Hexosomes were found to stimulate significantly stronger humoral immune responses than CAF04, but much weaker cellular immune responses. The authors additionally varied the charge of hexosomes by adding charged lipids and found that charge was not responsible for the difference between hexosomes and liposomes. It was therefore suggested that other factors such as the particle structure might be responsible for the different biological performance of the two carriers [99].

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4. Cubosomes

Liquid crystalline phases, also called mesophases, share features of both solid crystals and melts. Many materials form a liquid crystal phase over a temperature range during the process of melting. These materials consequently melt over a temperature range in contrast to the abrupt transition generally expected for the solid-melt phase transition. The stability of such liquid crystals is controlled by temperature and they are referred to as thermotropic mesophases. Materials that form liquid crystals upon addition of solvent are called lyotropic mesophases. Lyotropic mesophases are formed by self-assembly of amphiphilic molecules by the addition of water and are characterized by having a thermodynamically stable morphology with long-range order in one, two or three dimensions [1,2]. Lyotropic liquid crystals generally exhibit no ordering on the atomic scale (melt) but have molecular ordering on a larger length-scale leading to a well-defined structure (crystal) [2].

The first report of dispersed particles of the bicontinuous cubic phase is from Patton and Carey in 1979 [3]. In their study on fat digestion, they discovered that particles of the bicontinuous phase formed when simulated gastric fluid was combined with bile salts and lipases [3]. A decade later, Larsson coined the term cubosomes to describe particles of the bicontinuous cubic phase. Larsson has following been credited with discovering that cubic phases can exist as bulk macroscopic phases and dispersed into particles with the same structure as the bulk phase [4].

4.1. Self-assembly of particles from amphiphilic molecules

The hydrophobic end of amphiphilic lipids is often referred to as the tail, and the hydrophilic end is referred to as the head. The principal driving force for self-assembly of amphiphilic lipids in polar solvent is the so-called hydrophobic effect. The hydrophobic effect refers to the minimisation of interactions between the non-polar hydrocarbon tails of the amphiphiles and the surrounding water [5]. It mainly stems from the strong hydrogen bonding between water molecules, which causes water to expel non-polar solutes that are incapable of forming hydrogen bonds [6]. The morphological structure of the amphiphile self-assembly aggregates is determined by factors such as the molecular shape and

concentration of the amphiphilic molecule as well as external factors such as temperature, pressure and pH [1,6].

The critical packing parameter (CPP), also known as the shape factor, is used to describe the molecular shape of amphiphiles. CPP is defined in Equation 1:

$$CPP = \frac{v}{a_0 \cdot l_c} \qquad \text{(Eq. 1)}$$

where v is the hydrophobic tail volume, a_0 the area of the head group/tail group interface and l_c the length of the hydrophobic tail in the molten state [2,6], as illustrated in Figure 17.

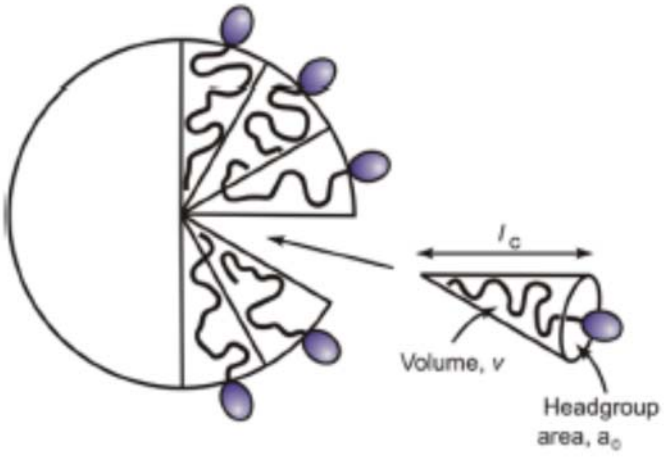

Figure 17. Illustration of the parameters v, a_0 and l_c determining the CPP used to describe the shape of amphiphiles. The CPP connects the shape of the amphiphile to the curvature of the lipid/water interface favoured by the system. v is the volume of the lipid tail, a_0 the head group area and l_c the length of the hydrophobic tail in the molten state. Reprinted with permission from [7]. *Copyright (2016) American Chemical Society.*

The CPP connects the shape of the amphiphilic molecules to the curvature of the lipid/water interface favoured by the system. Oil-in-water systems with membrane curvature towards the chain region are referred to as Type 1 or normal phases, whereas water-in-oil systems with curvature towards the water region are referred to as Type 2 or inverse phases. A rule of thumb suggested by Kaasgaard and Drummond is that Type 1 phases occur where $CPP < 0.5$ and Type 2 where $CPP > 1$. Planar phases giving rise to lamellar structure arise when $0.5 < CPP < 1$ [6], all as illustrated in Figure 18. Others suggest that Type 1 is likely to occur when $CPP < 1$ and Type 2 when $CPP > 1$ [2,8].

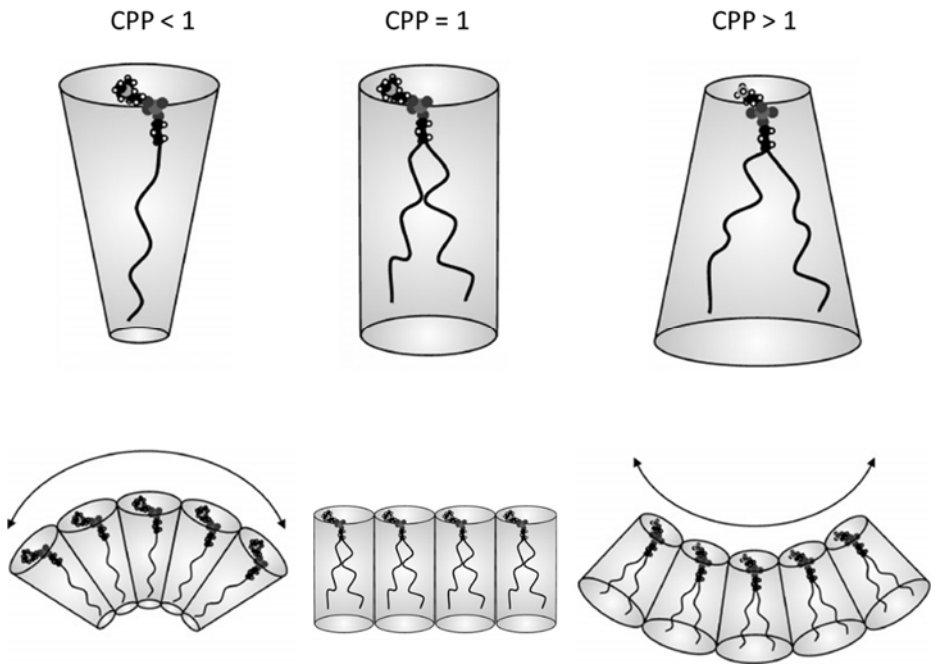

Figure 18. The effect of the molecular shape of amphiphiles on their packing structure. Oil-in-water curvature results from CPP < 1, no curvature from CPP = 1 and water-in-oil curvature when CPP > 1. Figure adapted with permission from [8] *© IOP Publishing. All rights reserved.*

It should be noted, that while this model is useful for explaining observed amphiphile phase behaviour, it is difficult to use predictively for novel systems since v, a_0 and l_c are difficult to estimate. The parameters are furthermore not constants, but depend on the local environment. Changes in temperature, pressure, pH, salt concentration and amphiphile concentration all affect the parameters [5,6].

4.2. Lyotropic liquid crystals

Various systems of amphiphiles in solvent can express a wide range of lyotropic liquid crystal structures as reviewed elsewhere [2,6]. The lamellar, hexagonal and cubic phase will be presented here.

4.2.1. Lamellar liquid crystals

The lamellar mesophase (denoted L_α) has a simple one dimensional crystal lattice organising the amphiphiles into a planar sheet-like structure. The amphiphilic molecules

are arranged such that the heads are in contact with water and the tails face each other. This shields the tails from contact with water by forming a double layer structure. Lamellar sheets can be stacked on top of each other forming a layer of amphiphiles with head-head and tail-tail intersections [6], as illustrated in Figure 19.

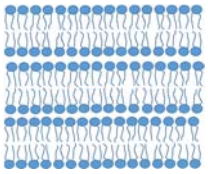

Figure 19. Illustration of the structure of stacked lamellar amphiphile bilayers.

4.2.2. Hexagonal liquid crystals

The hexagonal phase consists of densely packed cylindrical micellar columns arranged in a hexagonal two dimensional pattern. Bulk hexagonal mesophases exist in the normal, H_1 ($CPP = 0.5$), and inverse, H_2 ($CPP > 1$), configuration, as illustrated in Figure 20. In the H_1 phase, a continuous water domain separates hydrophobic cylinders, whereas in the H_2 phase, a continuous hydrophobic domain separates cylindrical water channels (section 4.1). In both cases the hydrophobic tails are molten [2].

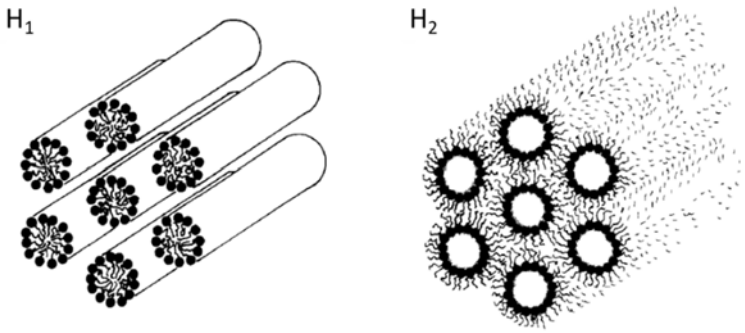

Figure 20. Illustration of the structure of the normal hexagonal phase (H_1) and the inverse hexagonal phase (H_2). Adapted from [9] *with permission from Elsevier.*

The H_2 mesophase can exist as dispersed particles in excess water called hexosomes [10]. The inverse hexagonal mesophase is strongly affected by packing constraints because the distance from the centre to the unit cell boundary of the hexagonally packed rods is not constant thus creating a void which must be filled uniformly (illustrated in Figure 21). In single amphiphile systems, where all hydrophobic tails are of equal length, this causes

strong packing frustration as lipid tails are forced to tilt or stretch away from their preferred conformational states to fill the voids. Strategies that reduce this packing frustration therefore favour the formation of the H_2 mesophase. The void volume is proportional to the water channel diameter and factors such as low water content and high CPP (e.g. through elevated temperature, hydrophobic chain unsaturation and decreased chain length) thus favour formation of the H_2 phase [6,8].

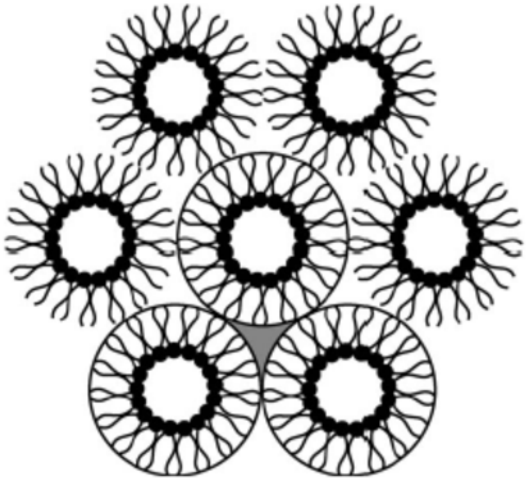

Figure 21. Illustration of packing frustration in the inverse hexagonal phase. The hexagonally packed cylindrical rods create a void indicated by the grey area. The area must be filled by additives or by energetically costly bending and stretching of the lipid chains. Reprinted from [6] *with permission from the PCCP Owner Societies.*

4.2.3. Cubic liquid crystals

The surface structure of cubic phases has a triply periodic saddle shaped geometry that can be modelled mathematically as infinite periodic minimal surfaces (IPMS) [2,6]. Minimal surfaces are surfaces in which the principal curvatures are equal in magnitude and opposite in sign giving a mean curvature of zero at all points. Principal curvatures are a mathematical concept describing the signed maximum and minimum curvature of the line obtained by the intersection between the surface and any plane containing the normal vector to the surface at the point in question (Figure 22) [11].

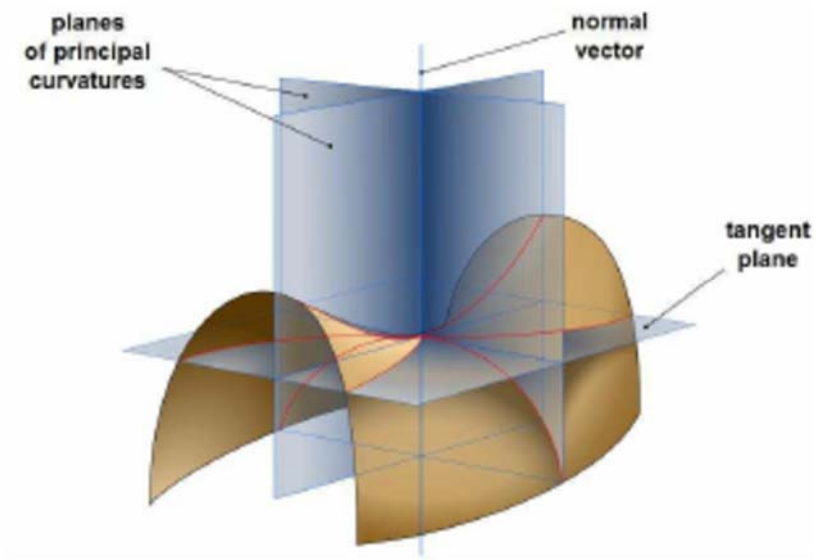

Figure 22. Principal curvatures of surface. The principal curvatures of the golden surface in a specific point are the signed curvatures of the red lines in the intersection between the surface and the planes of principal curvature. The planes of principal curvature contain the normal vector to the surface in the specific point and are rotated to obtain maximal signed curvature of their intersection with the surface. Figure reprinted from [12].

IPMS are infinite extensions of minimal surfaces giving a periodic structure. Three IPMS structures have been observed in amphiphile/water systems: the gyroid (G), diamond (D) and primitive (P) corresponding to the *Ia*3*d*, *Pn*3*m* and *Lm*3*m* space groups, respectively. These structures are illustrated in Figure 23 [6,8].

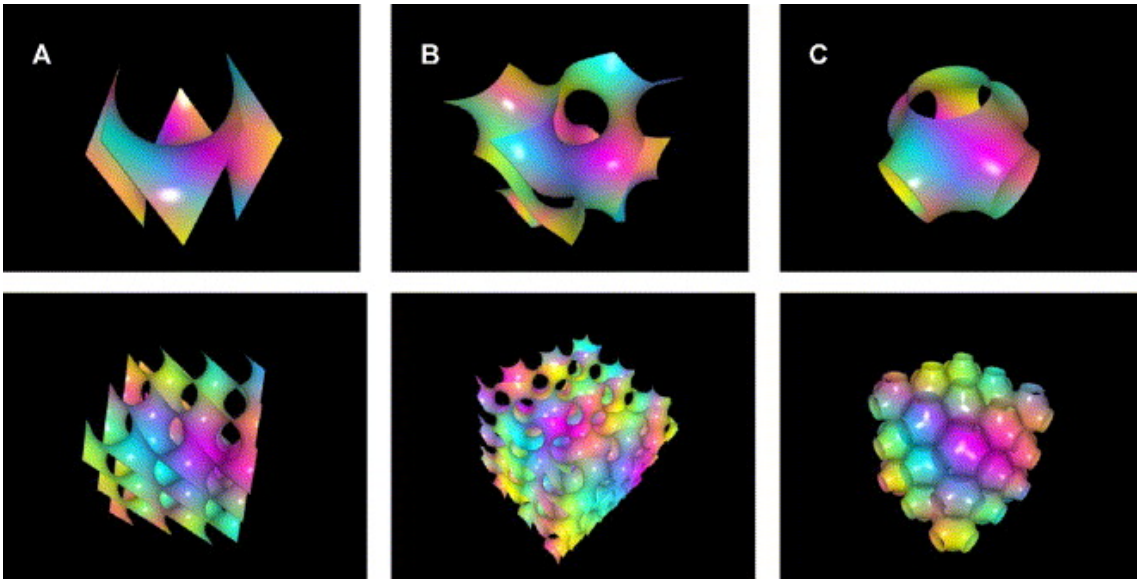

Figure 23. Structure of cubic phase minimal surfaces: A) diamond, B) gyroid and C) primitive. The top panels show the unit cell of the surface and the bottom panels show extensions of these forming cubic phase periodic minimal surfaces. Reprinted from [1] *with permission from Elsevier.*

Cubic phases are bicontinuous mesophases meaning that they consist of two immiscible liquids that form continuous interpenetrating phases. These phases are formed by a single bilayer membrane similar to the lamellar membrane, but warped into the saddle shaped geometries described above [2]. As for the hexagonal mesophase, the cubic mesophase can exist as Type 1 (V_1), with two interwoven cubic networks of hydrophobic channels surrounded by water ($CPP \sim 2/3$), or as Type 2 (V_2), with two interwoven cubic networks of water channels surrounded by a hydrophobic phase of opposing hydrocarbon chains ($CPP > 1$). The three cubic mesophases have also been described as a set of monolayers, giving rise to their names G, D and P. The G mesophase consists of a pair of 3-connected (gyroid) "Y" networks, the D of 4-connected "diamond" networks and the P of identical primitive 6-connected simple cubic networks [2].

Inverse bicontinuous cubic phases have received growing interest for their potential as drug delivery system [13]. They have a porous structure [8] with highly tortuous water pores of a diameter typically close to 5 nm [14] and are thermodynamically stable, lasting indefinitely if the amphiphile is not degraded [15]. Hydrophobic and hydrophilic actives can be incorporated in the lipid and water phases of the V_2 mesophase, respectively, allowing incorporation of a wide variety of actives. However, when substances are incorporated above a certain concentration, they can disrupt the cubic structure of the lipid

bilayer. This limits the amount of actives that can be loaded into cubosomes. Generally, lipophilic drugs will favour transformation to the H_2 phase and hydrophilic drugs will favour transformation to the lamellar phase [14]. Furthermore, small changes in temperature can cause transitions between the different cubic phase structures and transitions to e.g. lamellar or hexagonal phases can result from larger temperature changes [2].

The V_2 phase has been shown to be able to protect actives such as vitamins and proteins from external challenges (e.g. enzymatic or oxidative degradation). It additionally possesses the potential for sustained release as diffusion of water-soluble actives is delayed by the tortuosity of the water channels [14,15]. Bulk cubic phases are highly viscous and may be nearly solid [2] making them impractical to handle and unsuitable for injection [1]. Colloidal dispersion of the V_2 phase, cubosomes, with the same internal substructure as the bulk "parent" phase can be obtained by various methods [1], as described in section 4.5. Cubosomes have been proposed to possess a similar capacity as the parent phase for sustained release of actives [15] and this has later been verified experimentally for the release of OVA by Rizwan et al. [16]. However, there are also studies showing that cubosomes do not offer sustained release [17]. Rizwan et al. discussed that the sustained release of OVA probably in part is related to OVA being a relatively large (45 kDa) and surface active molecule because small molecule drugs are known to be released quickly from cubosomes [16].

4.3. Cubic phase forming lipids

As evident from the discussion in section 4.1, certain combinations of hydrophilic head groups and hydrophobic tail groups are likely to provide a certain phase behaviour. A range of lipids have been identified to form non-lamellar liquid crystals. The most studied lipids are monoolein and phytantriol, whose chemical structures are shown in Figure 24 [5]. Monoolein has low solubility in water but can swell in aqueous medium [13]. It forms V_2 phase at low temperatures in excess water and transforms to H_2 phase and inverse micellar phase at elevated temperature (Figure 25, section 4.5). Although, phytantriol has quite different chemical structure from monoolein and is an oil rather than a wax at room temperature, the phase behaviour of the two lipids is similar [18].

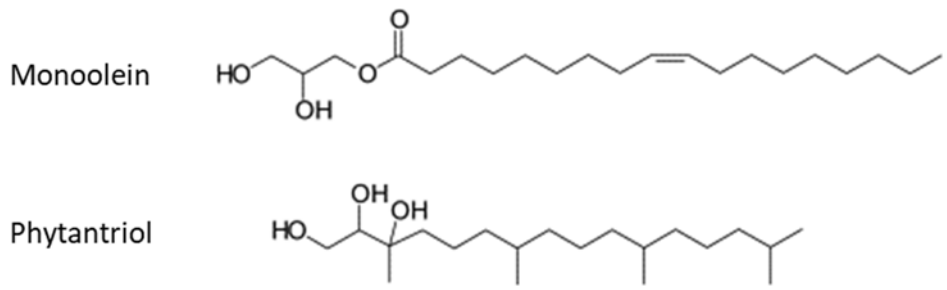

Figure 24. Chemical structures of monoolein and phytantriol. Monoolein and phytantriol are commonly used lipid amphiphiles to form non-lamellar lyotropic mesophases. Adapted from [19] *with permission from Elsevier.*

Monoolein is a naturally occurring lipid in the GI-tract being a product of the digestion of triglycerides. It is prepared commercially for use as a food emulsifier, although with limited purity. Monoolein has several desirable properties for vaccine purposes. It has been awarded with the "generally recognised as safe" status, is non-toxic, biocompatible and biodegradable [13,14]. Monoolein can be hydrolysed by acid and certain enzymes, and the presence of unsaturation further renders it susceptible to oxidative degradation [5].

Phytantriol is used commercially in the cosmetics industry and has the advantage that the purity of commercially sourced phytantriol is higher than that of commonly used monoolein sources (e.g. food additives sourced from Danisco such as Dimodan® MO 90/D used in this PhD). However, while it has greater purity, small amounts of impurities have been shown to strongly affect its phase behaviour [18]. Like monoolein, phytantriol is a biocompatible lipid regarded to be safe [20]. However, since phytantriol has neither ester bonds nor unsaturation, it is more resistant to chemical degradation than monoolein [5].

4.4. Structural characterisation of dispersed liquid crystals

Dispersed liquid crystals have been characterised using a range of techniques as reviewed by Boyd et al. [18]. The most popular techniques are small angle x-ray scattering (SAXS) and cryogenic transmission electron microscopy (cryo-TEM) [18].

The best way to determine the lattice structure of dispersed liquid crystals is through SAXS [2,5]. The crystalline lattice of liquid crystals diffracts x-rays in radially symmetric two dimensional patterns characteristic of the structural organisation of the lattice. These are

converted to one dimensional patterns in which the relative peak positions (Bragg peaks) allow determination of the crystal lattice structure of the sample. The position of the peaks additionally provide information about the size of the lattice [5,18]. The relative Bragg peak distances expected from selected liquid crystal structures are shown in Table 2. At least 4 peaks in a spectrum should be obtained for confident statements about the symmetry aspect of the mesophase which is used to determine the structure [2].

Table 2. Relative spacing between Bragg peaks in SAXS diffraction patterns from selected liquid crystal phases. Table compiled from Hyde [2] *and Wörle et al.* [21].

Mesophase	**Descriptor**	**Peak ratios**
Lamellar	L_α	$1: 2: 3: 4: 5: 6$ ...
Hexagonal	H_1, H_2	$\sqrt{1}: \sqrt{3}: \sqrt{4}: \sqrt{7}: \sqrt{9}: \sqrt{12}$ ...
Bicontinuous cubic	G (*Ia3d*)	$\sqrt{6}: \sqrt{8}: \sqrt{14}: \sqrt{16}: \sqrt{18}: \sqrt{20}$ ...
	D (*Pn3m*)	$\sqrt{2}: \sqrt{3}: \sqrt{4}: \sqrt{6}: \sqrt{8}: \sqrt{9}$ ...
	P (*Lm3m*)	$\sqrt{2}: \sqrt{4}: \sqrt{6}: \sqrt{8}: \sqrt{10}: \sqrt{12}$ ...

SAXS provides excellent information, but also has limitations. Amphiphiles used to make liquid crystals are typically organic molecules consisting of atoms with weak electron density leading to low scattering intensity. The signal intensity will be further lowered by the low particle concentrations commonly used in the dispersions and therefore conventional SAXS instruments require hours of acquisition. Synchrotron radiation sources can greatly increase x-ray flux thus reducing necessary acquisition times to seconds and allow characterisation of structural changes in response to stimuli [5,18]. Another limitation of SAXS is that it does not provide information on single particle structure. Therefore, in systems with a mixture of particle morphologies, SAXS may not lead to identification of all particle systems present [10].

Cryo-TEM allows direct visualisation of particles dispersed in aqueous medium. Regular electron microscopy techniques operate in vacuum and require the removal of solvent. However, since lyotropic mesophases only form in the presence of solvent (usually water), this would result in loss of the internal structure of the mesophase. It is therefore necessary

to vitrify the samples in order to retain the structure. Cryo-TEM relies on almost instant freezing of a thin film of water in which the particles of interest are dispersed. Equilibrium structures are thus trapped in the ice providing a "snap shot" of the particles in dispersion [5,18]. One of the main advantages of cryo-TEM is the ability to visualise single particles. This allows identification of co-existing particle types with different internal structures including those that may not be visible on SAXS measurements [10]. Cryo-TEM studies have revealed that cubosomes often co-exist with vesicles and commonly have vesicles attached to their surface. These have been speculated to stabilise the cubosomes or to be precursors in a kinetic cubosome forming process. A major disadvantage of cryo-TEM is the inability to study dynamic changes [18].

Cryogenic field emission scanning electron microscopy (cryo-FESEM) has recently been suggested for characterisation of dispersed liquid crystals [1]. Cryo-FESEM provides three dimensional information on particle structure in contrast to cryo-TEM.

4.5. Production of cubosomes

Cubic mesophase can exist in three forms: precursors that form cubosomes upon hydration, macroscopic bulk gel and particle dispersion (cubosomes) [22]. Cubosomes can be produced by the top-down strategy through dispersion of bulk inverse bicontinuous cubic phase gel or by the bottom-up strategy in which dispersed cubosomes are created directly or from precursors [5]. The top-down strategy is the most common method for producing cubosomes. In this method, amphiphiles are first hydrated to drive their self-assembly into an inverse cubic phase bulk gel in a fully swelled state. A large energy input is subsequently required to break up the gel into particles. This is often done through high-pressure homogenisation, ultrasonication or high speed shearing and it is usually necessary to add steric stabilisers to obtain a stable dispersion of submicron cubosomes [5]. The top-down preparation of monoolein- or phytantriol-based cubosomes typically results in particles of 200-300 nm in diameter with a polydispersity index (PDI) of 0.1-0.4 which can be stable against aggregation for up to one year [5,16]. However, the prerequisite formation of bulk gel and the methods to disperse it may be difficult to scale up and the high-energy dispersion of bulk gel generates heat which might degrade sensitive components added to the bulk phase [5,16,23].

The bottom-up approach of hydrotrope dilution was first introduced in 2001 by Spicer et al. to overcome the drawbacks of the top-down approach [24]. In this method, a cubosome precursor mixture of monoolein dissolved in ethanol was diluted with water containing the steric stabiliser pluronic 127 to generate cubosomes with minimal energy input [24]. The critical parameter to this method was the use of a hydrotrope. Hydrotropes are amphilic materials without surfactant properties and are thus incapable of surfactant phase behaviour such as micelle formation. They characteristically have short compact hydrophobic tails in contrast to the longer tails of surfactants [25] and prevent liquid crystal formation only at high hydrotrope concentrations [23]. The ternary phase diagram for the monoolein/water/ethanol system can be used to design a cubosome forming dilution process (Figure 25). A single phase isotropic liquid of low viscosity is present in the region of the ternary phase diagram where the ethanol concentration is above 10 %. This liquid forms cubosomes upon dilution with water into the areas between the cubic phase regions and the isotropic liquid region. An example of the process is illustrated with the “dilution path” in Figure 25 [24]. The hydrotrope dilution method was later shown to be applicable for a range of other amphiphile/water/hydrotrope systems as well [16].

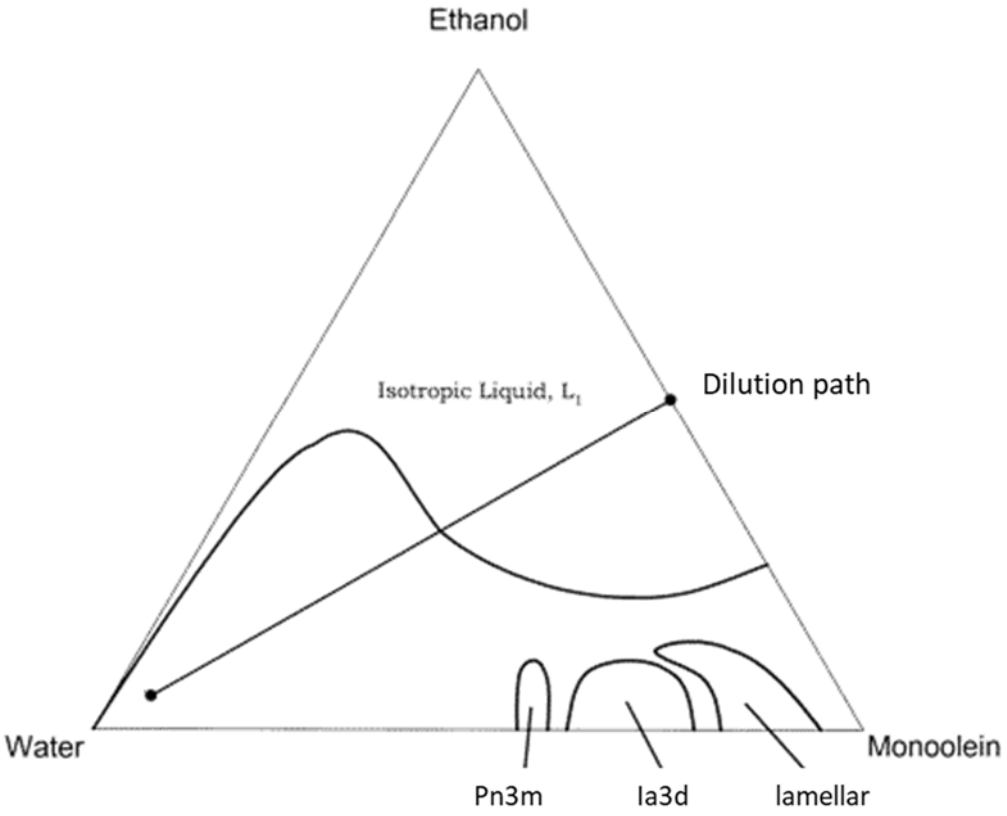

Figure 25. Ternary phase diagram of the water/ethanol/monoolein system at 25°C. The system exhibits four single phase regions including the cubic mesophases of structure Pn3m and Ia3d and the lamellar mesophase. These mesophases extend as high as 10 % ethanol. The large region of isotropic liquid provides flexibility for the formulation of precursors to form cubosomes or cubic gel upon dilution in water. An example of the generation of cubosomes by dilution with water is indicated with the dilution path line. Adapted with permission from [24]*. Copyright (2001) American Chemical Society.*

The dilution-based approach avoids laborious fragmentation and is more efficient at generating small particles than the fragmentation approach. In addition, cubosomes prepared through dilution with appropriate stabilisers can show long term colloidal stability [26]. However, designing a dilution-based process is complicated as it relies on knowledge of the ternary phase diagram (amphiphile/water/hydrotrope) as well as the effect of additives such as stabilisers and biological actives. Furthermore, the addition of a hydrotrope complicates the cubosome preparation as the individual components often will have different solubilities in the hydrotrope. The use of a hydrotrope also has the potential drawback that the hydrotrope could give rise to adverse effects upon administration [5,26],

although this drawback may be alleviated by drying the precursor mixture as will be described in section 4.6.

4.6. Spray drying cubosomes

Most vaccines are sensitive to temperature fluctuations, heat and/or freezing. Live vaccines are generally more sensitive to potency loss at elevated temperatures, whereas inactivated and subunit vaccines often tolerate moderate heat exposure. These vaccines are, however, often sensitive to freezing. This is mostly due to adjuvants such as alum, which might collapse upon freezing thus lowering its adjuvant effect [27]. The cold chain was developed to accommodate both of these types of vaccines. Unfortunately, cold chain problems are common and have been documented in all countries where temperature monitoring has been conducted, including developed countries [27]. Formulation of vaccines in powder form can improve their stability and spray drying has been suggested to be an ideal drying method for some vaccines [28]. Ohtake et al. reported a substantial improvement in the stability of a commercial live attenuated measles vaccine when using spray drying compared to the commercial lyophilised product. The loss of activity during drying was also reduced compared to lyophilisation [29]. Powder forms of vaccines furthermore avoid the need to transport bulk water and gives access to a wider range of applications [22,29].

Cubosome precursor powders have been produced by freeze-drying [30] and spray drying [22,31–33]. Spray drying is a method consisting of three steps: atomisation of the liquid feed, evaporation of the solvent and collection of the powder [34]. The spray drying process employed during this PhD project is illustrated in Figure 26, but many variations are possible as reviewed elsewhere [35]. The high temperature of the drying air used for spray drying has been a cause of wonder regarding how heat-labile biologicals can resist heat induced denaturation during spray drying. The explanation is related to the constant evaporation and short transit time through the hot drying chamber. At the early stage of the drying process where the air is hot, the droplet surface remains saturated with moisture. Moisture constantly and rapidly evaporates from the droplet surface and the droplet is therefore maintained at the wet-bulb temperature, which is considerably lower than the temperature of the surrounding air. At the later part of the drying process, the droplet surface can no longer remain moisture saturated and the evaporation driven temperature difference between the droplet and the surrounding air decreases. However, at this time the

biological is primarily in the solid state where it is more tolerant to heat. The temperature of the surrounding air has at the same time been reduced due the evaporation that has already taken place [34]. The maximal temperature the dry biologicals might experience is the therefore the outlet temperature of the spray dryer [35] and thermal denaturation of biologicals is consequently rare [34].

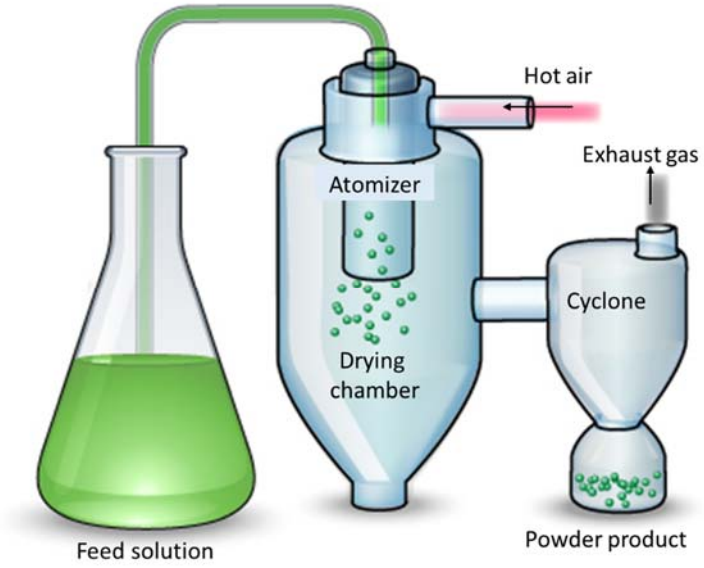

Figure 26. Schematic of the spray drying process used for this PhD project. A feed solution is pumped into a drying chamber and dispersed into a fine mist by an atomising air flow. A hot air flow dries the particles in the drying chamber and carries them into the cyclone. In the cyclone, particles are deposited by centrifugation and fall down to a collecting chamber below. Adapted from Paper I.

Spray drying has received growing interest due to its relative simplicity, scalability and cost-effectiveness [28]. Engineering of the powder particles is possible as reviewed elsewhere [28] and sterile spray drying is also feasible [36]. In relation to this PhD, an important advantage of spray drying is that it can directly form a fine flowable powder [34] making it possible to fill it into microcontainers without further processing (Paper I). This is in contrast to freeze drying, which tends to form a cake or a cotton-like substance which would need to be broken into particles through a secondary process [34].

Motivated by the extended applicability of dry powder forms of cubosomes, Spicer et al. developed a spray drying method to produce cubosome precursors [22]. Solid monoolein can theoretically be fragmented into small particles, but the sticky waxy nature of the lipid

causes powder cohesion. Stabilisers must therefore be added to encapsulate the lipid and reduce powder cohesion [23]. Spicer et al. first spray dried a mixture of monoolein, water and hydrophobically modified starch. When using a low MW starch (84 kDa), a powder yield of 78 % w/w was obtained and the spray drying process was reported to result in a non-cohesive powder of cubosome precursors encapsulated in starch shells. The powder was easily reconstituted into colloidally stable cubosomes upon addition of water, with the hydrated starch now acting as steric stabiliser of the dispersed cubosomes. However, the monoolein/water/starch system was difficult to spray dry because cubosomes formed immediately upon hydration of monoolein and starch. This caused a need for high energy processing of the spray drying feed and frequent clogging of the spray drying nozzle [22,23]. The hydrotrope dilution method (presented in section 4.5) was therefore adapted for spray drying and ethanol was used as hydrotrope to dissolve the liquid crystals. As starch is insoluble in ethanol, it was replaced with dextran [22] which has finite solubility in mixtures of ethanol and water [37]. Mixing ethanolic monoolein with aqueous dextran resulted in a low viscosity emulsion that could easily be spray dried to form a stable flowable powder of fine particles. These were thought to consist of monoolein encapsulated in dextran shells. The powder could be easily hydrated to form a dispersion, but tended to agglomerate over time although it could easily be redispersed. The authors explained the better ability of starch to provide colloidal stabilisation to be related to its favourable balance of hydrophilicity and hydrophobicity helping it interact with both water and lipid. Dextran on the other hand is mainly hydrophilic and thus does not interact much with the lipid bilayers [22].

The method of Spicer et al. [22] was later adapted by Nielsen et al. [31] to produce cubosomes carrying the protein OVA. Nielsen's method was adapted in this PhD project to produce a dry powder vaccine formulation consisting of cubosomes with OVA as model antigen and Quil-A as adjuvant. OVA has been reported to resist ethanol induced aggregation at ethanol concentrations up to 20 % w/w in the ternary water/ethanol/OVA phase diagram at OVA concentrations below ~5 % w/w and a temperature of 20-40ºC [38]. For this reason, an ethanol concentration of 20 % w/w in water disregarding the presence of monoolein, dextran, OVA and Quil-A was used in this thesis. This should not cause aggregation of the OVA since the OVA concentration in the mixture used here is several orders of magnitude below 1 %. At the same time, it is a sufficient concentration to ensure

that ethanol will exert its hydrotropic effect on monoolein providing a low viscosity liquid for the spray drying feed [22].

A limited number of microcontainers can be loaded into a mouse sized capsule. This was an important formulation constraint, since it limits the dose of cubosome precursor powder that can be administered orally to mice in microcontainers. For oral vaccination, antigen doses are generally required to be 10-100 times higher than the necessary dose for injected vaccine [39]. The OVA concentration in the spray drying feed was therefore increased to obtain a high load of antigen in the powder, and thus facilitate later use of the formulation in microcontainers carrying an appropriate antigen dose in a mouse capsule. The spray drying process and the *in vitro* and *in vivo* evaluation of the produced cubosome precursors as vaccine system is presented in Chapter 6 (Paper I).

Typical effects of varying different spray drying parameters on characteristics of the spray dried powder are summarised in Table 3, which can be used as a rough guide when developing a spray drying process. However, due to heterogeneity in the behaviour of various substances during drying, optimisation is usually done in a trial-and-error manner [35,40]. The outlet temperature cannot be controlled directly, but depends on the other parameters (Table 3) [35,40].

Table 3. General effects of spray drying parameters on process parameters (outlet temperature and yield) and powder product parameters (particle size and humidity). ↑, ↑↑ and ↑↑↑ indicates mild to strong increase and ↓, ↓↓ and ↓↓↓ mild to strong decrease. Parentheses indicate eventual effects. Table modified from [40].

Parameter / Dependence	Aspirator rate ↑	Air humidity ↑	Inlet temperature ↑	Atomising air flow ↑	Feed rate ↑	Heat capacity of solvent ↓	Concen-tration of feed ↑
Outlet temperature	↑↑	↑	↑↑↑	↓	↓↓	↑↑↑	↑↑
Particle size	-	-	-	↓↓↓	(↑)	(↓)	↑↑↑
Humidity of product	↑↑	↑↑	↓↓	-	↑↑	↓↓↓	↓
yield	↑↑	(↓)	(↑)	-	(↓↑)	↑↑	↑

The most important process parameters for spray drying are the drying air inlet temperature, outlet temperature, flow rate of the drying air (aspiration rate), atomising air flow rate and the residence time of the droplet in the drying chamber [34]. In paper II (Chapter 7), the effect of varying the inlet temperature, aspiration rate, feed rate and atomising air flow rate were evaluated. The residence time was not included as a variable parameter since it was not possible to control directly. The outputs of interest were the outlet temperature, powder yield, residual moisture, reconstitution properties, and the size and morphology of the particles after rehydration. The temperature of the inlet air directly influences the airs ability to dry and the rate of evaporation. The aspiration rate is directly influential on the obtained powder yield as increased flow rates increase rotational speed and thus the sedimentation force of particles in the cyclone (Figure 26). However, it also increases the speed at which droplets move through the drying chamber, decreasing drying time and thus possibly increasing the residual moisture. The atomising air flow rate controls the droplet size with higher flow rates giving smaller droplets. Smaller droplets give shorter drying times and generally provide smaller powder particles, however, its effect on the size of the rehydrated particle was unknown [35]. The effect of the process parameters on particle morphology and reconstitution properties was also unknown. The architecture and morphology of spray dried particles are controlled by the feed composition and by the distribution of compounds in the particles. The latter is affected by a number of complex phenomena related to the composition of the feed and by the rate of evaporation from the droplet (i.e. the drying rate). A “slow drying rate”, in which the diffusional motion of the solute is fast compared to the radial velocity of the receding droplet, allows diffusional rearrangement of molecules during drying and often leads to the formation of solid particles. Conversely, when the droplet surface recedes faster than the diffusional speed of the solutes, the solutes will concentrate on the droplet surface leading to the formation of a shell [28]. Ingvarsson et al. have previously spray dried pre-formed liposomes in suspension and found that the spray drying process affected the morphology of the reconstituted liposomes and that a fast drying rate was critical to preserve the liposome structure after drying [41].

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5. Microcontainers for oral vaccine delivery

Microcontainers are micro-fabricated polymeric cylinders in the micro size range that are closed at one end and open at the other end. A scanning electron microscopy (SEM) image of the microcontainers used for this PhD on a silicon chip is shown in Figure 27. They are fabricated with precisely controllable dimensions with the negative epoxy photoresist SU-8 through a two-step photolithography process.

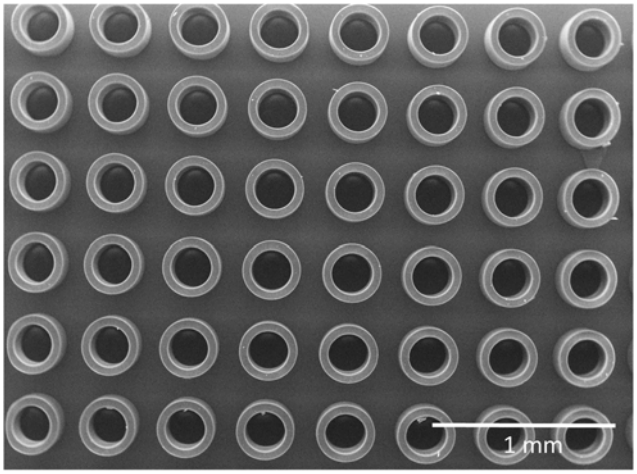

Figure 27. SEM image of the microcontainers used in this PhD project as seen from above. The microcontainers are produced in 25x25 arrays on a silicon wafer.

For this PhD, the concept of the microcontainers is that they can be filled with vaccine formulation and then sealed with a polymer lid, as illustrated in Figure 28. Many vaccine antigens are sensitive to the reduced pH and proteases in the stomach [1]. These can be protected by loading the vaccine into microcontainers and sealing them with a pH-sensitive lid that will remain stable in the pH range of the stomach, but will dissolve in the pH range of the intestine [2]. Microcontainers have previously been observed to be engulfed in the mucus of the rat intestine [3]. We speculated that this might help particles cross the mucosal barrier and may help lipid particles reach APCs before emulsification by bile salts.

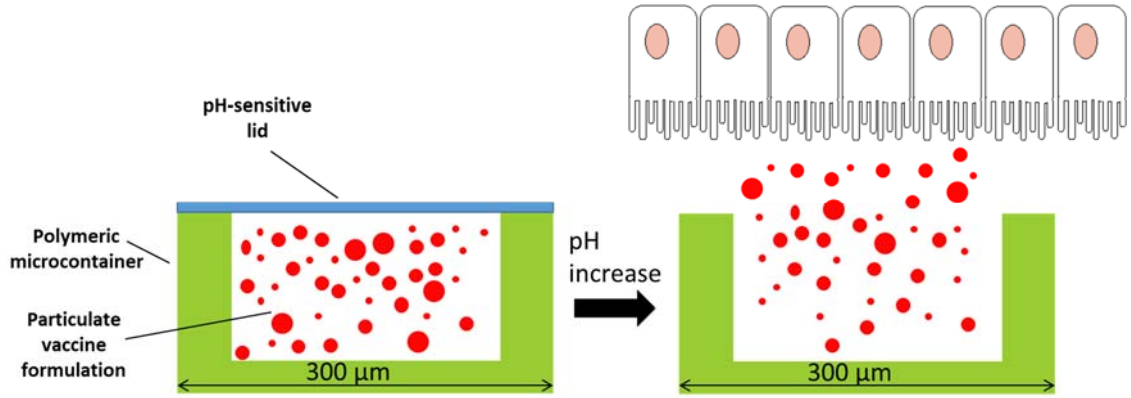

Figure 28. Concept of microcontainers for oral vaccine delivery. A powder formulation of the vaccine is loaded into the microcontainer and the microcontainer is then sealed with a pH sensitive lid. The pH sensitive lid remains intact at low pH (stomach) but dissolves at increased pH (intestine), thus protecting the vaccine from the harsh conditions of the stomach and releasing it in the intestine.

In this PhD study, a spray drying process was established to produce a model vaccine in powder form. The vaccine consisted of powder precursors of cubosomes with the adjuvant Quil-A and the protein ovalbumin as model antigen. *In vitro* evaluation of the particle morphology, size, zeta potential, particle stability in dispersion, antigen content and release was investigated as well as the stability of the antigen during dry storage of the powder at room temperature. The vaccine showed promising properties for vaccination, although the cubosomes needed a high energy input to disperse and had limited colloidal stability. Further evaluation *in vivo* showed that the vaccine was highly immunogenic after s.c. administration stimulating strong antigen specific humoral and cellular immune responses. However, when administered orally in powder form in a capsule, no immune response could be detected (Paper I, Chapter 6). It was speculated that optimisation of the spray drying parameters might improve the reconstitution or affect the structure of the particles after rehydration. A design of experiments approach was employed to evaluate the effect of the four spray drying parameters expected to be the most influential on the product: input temperature, aspiration rate, atomising air flow rate and feed rate (Paper II, Chapter 7).

Microcontainers have previously shown promise as oral delivery system *in vivo* in rats, as described in Chapter 1. However, they have never been used in mice and have never been evaluated for oral delivery of a vaccine. In Paper I (Chapter 6), we established that cubosomes are ineffective orally when delivered in a capsule. This was speculated to be due to degradation of the antigen by gastric acid and enzymes combined with emulsification

of cubosomes by bile salts. For that reason, microcontainers with pH-sensitive lids were considered promising for oral delivery of cubosomes. The effect of stomach fluid and intestinal fluid on unprotected cubosomes was evaluated *in vitro* and pH sensitive lids were designed to allow release of cubosomes in the small intestine of mice, but not in the stomach. Microcontainers with pH sensitive lids were evaluated *in vivo* as oral delivery system for powder precursors of cubosomes in a prime-boost setting as oral booster following s.c. injected prime (Paper III, Chapter 8).

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6. Paper I

Spray dried cubosomes with ovalbumin and Quil-A as a nanoparticulate dry powder vaccine formulation

Christoffer von Halling Laier, Blake Gibson, Marco van de Weert, Ben J. Boyd,

Thomas Rades, Anja Boisen, Sarah Hook, Line Hagner Nielsen

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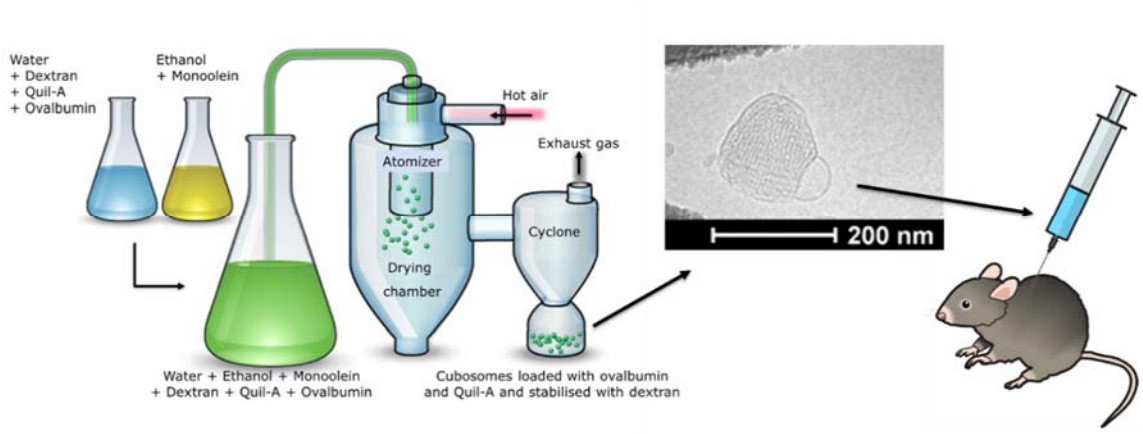
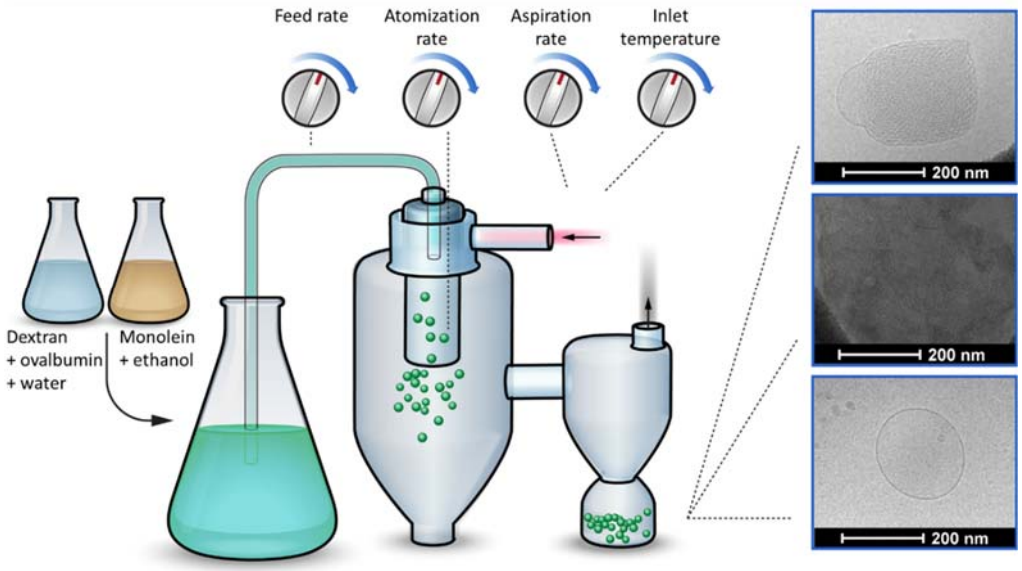


7. Paper II

Design of experiments on spray drying parameters for producing cubosome precursors

Christoffer von Halling Laier, Tommy Sonne Alstrøm, Mia Travers Bargholz, Pernille Bjerg Sjøltov, Thomas Rades, Anja Boisen, Line Hagner Nielsen

Technical note submitted to the European Journal of Pharmaceutics and Biopharmaceutics



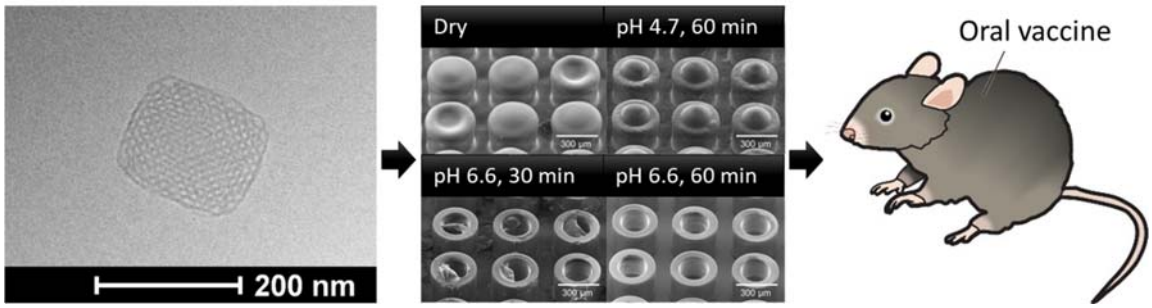
8. Paper III

Microcontainers for protection of oral vaccines, *in vitro* and *in vivo* evaluation

Christoffer von Halling Laier, Blake Gibson, Jorge Alberto S. Moreno, Thomas Rades, Sarah Hook, Line Hagner Nielsen, Anja Boisen

Manuscript in preparation

(planned for submission to the Journal of Controlled Release)



9. General discussion

The current study has focused on spray drying of a cubosome-based model vaccine and using microcontainers to deliver them orally. *In vitro* characteristics and the effect of varying spray drying parameters on the cubosomes were investigated and microcontainers were applied for oral delivery of the cubosomes using a pH-sensitive lid. Cubosomes and microcontainers were tested *in vivo* evaluating the potential of the system. In this section, important results of the studies will be discussed.

9.1. Re-dispersion and colloidal stability

For routine clinical administration of a vaccine, it is important that the vaccine formulation is easy to prepare. Any vaccine distributed in powder form and administered by injection should be able to reconstitute fully upon addition of water with gentle inversion of the container. Furthermore, as was established in sections 2.3 and 3.3.2.1, particles larger than 2-3 μm may be less efficiently taken up by intestinal M cells and APCs than smaller particles. For oral administration of a vaccine in powder form, it is consequently important that the powder can spontaneously reconstitute into dispersed particles when water is added.

In contrast to the easy re-dispersion reported by Spicer et al. [1] from a similar system (described in section 4.6), the cubosome precursors produced in this thesis suffered from poor reconstitution properties and limited colloidal stability. This could be related to the composition of the spray drying feed, the spray drying parameters, or to the large difference in the solids concentration used in the feed. The ratios of dextran (stabiliser) to monoolein (membrane forming lipid) used here and by Spicer et al. [1] are similar, and preliminary experiments to Paper I showed that the exact ratio is not crucial. However, OVA and Quil-A are both surface active molecules that might affect the surface properties of cubosomes and hence their colloidal stability. This is supported by the change in size and zeta potential of the particles observed when OVA and/or Quil-A was added (Paper I). The results of Paper II suggest that the only spray drying parameters that affect the powders ability to reconstitute are the inlet temperature and aspiration rate, with a high temperature and aspiration rate giving better reconstitution. These parameters also resulted in a high outlet

temperature and a common factor for all powders that reconstituted easily was an outlet temperature above 110ºC (Figure 29). This could indicate that a fast drying rate helps form powders that reconstitute easily. It has been suggested that a homogeneous distribution of a steric stabiliser on cubosomes helps form colloidally stable cubosomes [2]. It is therefore likely that it also provides easier reconstitution of the precursor powder and it was thus speculated in paper II that a fast drying rate is beneficial to obtain a powder that reconstitutes easily. Spicer et al. used a feed solution containing 40 % solids and an outlet temperature of 130ºC [1], whereas a solids concentration lower than 1 % was used in Paper II with outlet temperatures ranging from 36-132ºC. The effect of increasing the solids concentration of the feed was not investigated in Paper II, but this tends to increase the outlet temperature of the process (Table 3, section 4.6) because there is less solvent to evaporate.

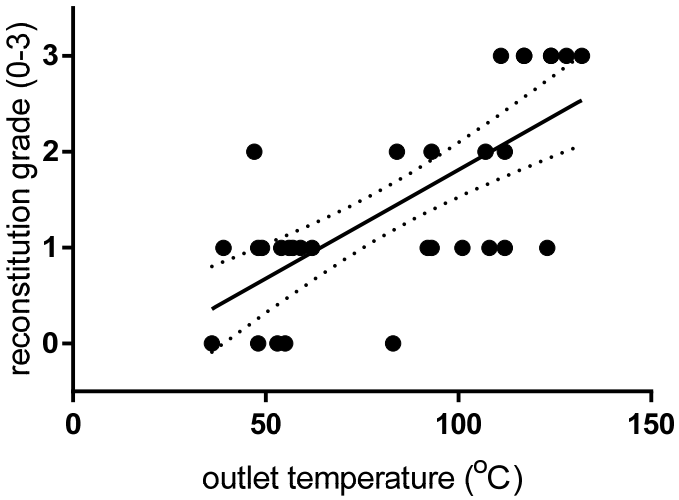


Figure 29. Effect of outlet temperature during spray drying on the ease of reconstitution of cubosome precursor powder graded from 0 (very poor) to 3 (excellent). A linear regression line (solid line, $r^2 = 0.51$) with 95 % confidence interval (dotted lines) is indicated.

While the outlet temperature was observed to be important for the reconstitution properties, it could only explain 51 % of the variation in the measured reconstitution grades in Paper II (Figure 29, unpublished figure). A better explanation of the poor re-dispersibility is that dextran is not a good steric stabiliser of cubosomes. This was suggested by Spicer et al. [1] due to the lack of a hydrophobic region on dextran, resulting in little interaction between dextran and the lipid membrane (section 4.6). Steric stabilisation of cubosomes is generally

necessary to prevent the particles from flocculating so they will remain as discrete colloidal particles [3]. The main elements required to obtain effective stabilisation are the volume and density of the hydrophilic stabilisation layer and the adhesion strength of the stabiliser onto the cubosome surface. Non-ionic block copolymers are often used for stabilisation of cubosomes and often comprise poly(ethylene glycol) or poly(ethylene oxide) to generate the hydrophilic stabilisation layer [3]. Pluronic 127 is considered the gold standard stabiliser and consists of the triblock copolymer poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide), which adsorbs to the surface of cubosomes [3,4]. This supports that dextran, due to its lack of a hydrophobic region, is likely not a good colloidal stabiliser of cubosomes. Adding the much better steric stabiliser Pluronic 127 to the spray drying feed may therefore result in a powder that is easier to reconstitute and shows better colloidal stability once in dispersion. The addition of Pluronic 127 does not obviate dextran, however, since an agent to reduce powder cohesion is still needed.

Flocculation of cubosomes upon reconstitution was an issue for the *in vivo* studies. With macroscopic aggregates, the dose given in each injection would be impossible to control, the particle size would be irreproducible and such large particles might not stimulate a proper immune response (sections 2.3 and 3.3.2.1). It was therefore necessary to reconstitute using glass beads and sonication. For consistency, this practice was also used for the *in vitro* studies in Paper I. Sonication was obviously not possible for formulation given orally in a capsule though. Since the *in vivo* results of Paper I showed a slightly stronger immune reaction of OVA and Quil-A in solution compared to the completely absent response of oral powder precursors of cubosomes with OVA and Quil-A in a capsule, it was speculated whether reconstitution could be the issue. Figure 30 (unpublished data) shows that the cubosomes powder not only did not reconstitute, but kept the gelatine capsule in a gelled state in unstirred buffer *in vitro* in contrast to the complete disintegration seen soon after hydration at 37ºC of a capsule without cubosomes.

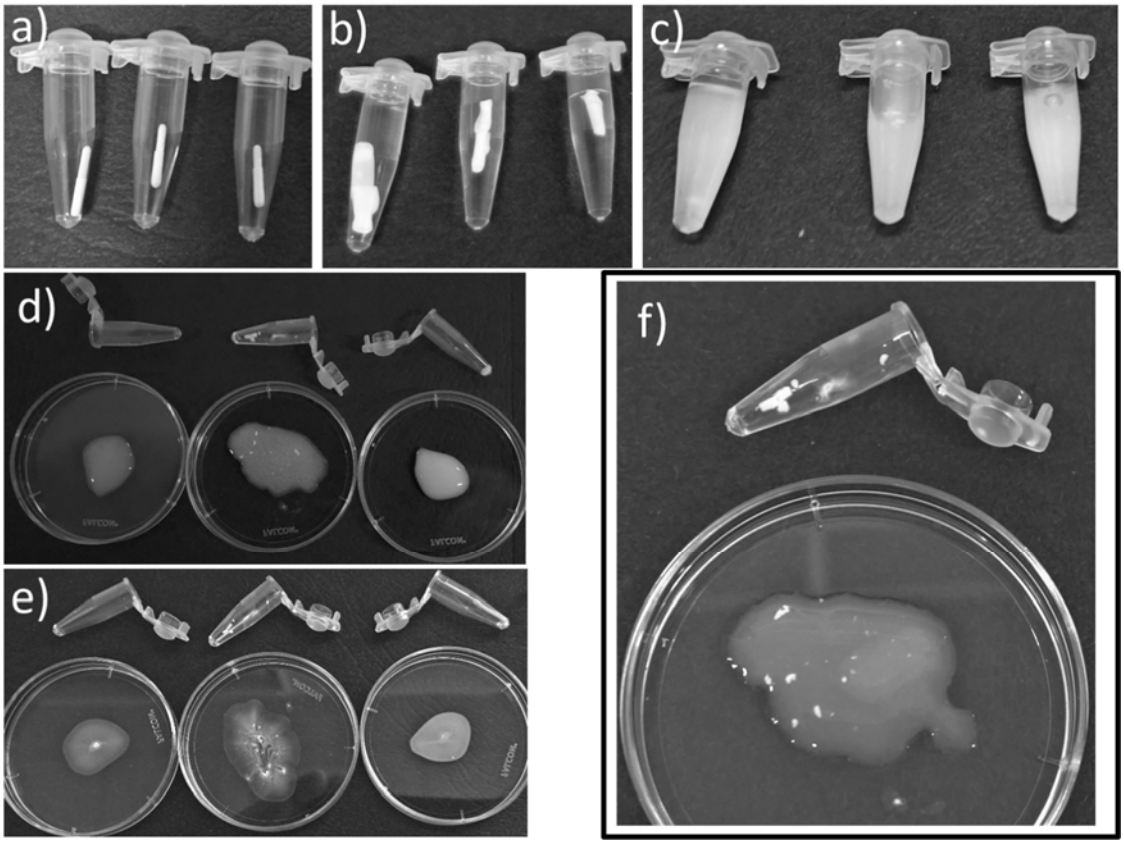


Figure 30. Oral capsules in 10 mM maleic acid buffer pH 6.6 in a volume of 200 µL at 37°C containing nothing (left), cubosomes with OVA and Quil-A (middle), and OVA+Quil-A (right). Pictures taken of a) dry capsules, b) after adding buffer c) after 10 min in buffer d) after 2 h in buffer and e) after letting the water evaporate. f) Close up picture of capsule with cubosomes after 2 h in buffer.

In the following *in vivo* study (Paper III, study 1), a control group of oral cubosomes with OVA and Quil-A was needed to evaluate the effect of microcontainers. This time, the cubosomes were dispersed prior to gavage to investigate if the lack of an immune reaction to the oral cubosomes seen in Paper I was a consequence of lacking reconstitution. This was partly confirmed by the study, although the results from dispersed oral cubosomes with OVA and Quil-A were highly variable and the immune stimulation was not comparable to that of s.c. injected cubosomes at a 10 times lower dose (Paper III, study 1). The result must be interpreted with caution, however, since administration of water can also increase the intestinal uptake of particles [5]. Solving the issue of re-dispersion is therefore important, but not sufficient to allow oral vaccination with cubosomes. Other factors that might affect the oral immune response include degradation of OVA by acid and proteinases in the GI-tract, emulsification of cubosomes by bile salts in the intestine, the ability of the vaccine to

cross the mucus barrier (section 2.2) and insufficient activation of individual APCs by both antigen and adjuvant (sections 2.1, 2.3 and 3.3.2.3). All but the last of these matters were attempted solved using microcontainers with pH-sensitive lids as presented in Paper III (Chapter 8) and will be further discussed in section 9.3. The issue of co-delivery of antigen and adjuvant to APCs is discussed below.

9.2. Co-delivery of antigen and adjuvant to APCs

Prior to the first *in vivo* study, it was established that 65 % of the OVA in the cubosome precursor powder was present in free soluble form after reconstitution and dispersion with glass beads and sonication. The remaining OVA was released within 24 h (Paper I). It was therefore interesting if Quil-A was also released quickly such that APCs would not be exposed to antigen before the immunopotentiator thus possibly being unable to stimulate a cellular immune response (section 3.3.2.3). As Quil-A is of surfactant nature and known to disrupt the cubosome structure at high concentration (unpublished data), it was considered possible that it might be incorporated into the lipid membrane. However, it is also very hydrophilic and consequently might also be expected to be present in the aqueous phase.

Measuring Quil-A release from cubosomes is not easy. Quil-A is a complex mixture of saponins with batch-to-batch differences (section 3.3.1.3) and low light absorption [6]. Bobbala et al. therefore developed a high pressure liquid chromatography with evaporative light scattering method to quantitate Quil-A and thereby enable measuring Quil-A release [6]. However, Quil-A tends to clog up HPLC columns and salts, lipids, dextran and OVA need to be removed for the method to work (unpublished data). Salts could be avoided by performing the release study in phosphate buffer without the added salts of the more physiological phosphate buffered saline. Since the MW of the saponins of Quil-A is in the range of 1500-2500 Da [7], it was anticipated that dextran, OVA (MW > 40 kDa, Paper I) and cubosomes could be separated from Quil-A by ultrafiltration through a 10 kDa membrane. However, although the concentration used was below the critical micelle concentration of Quil-A (section 3.3.1.3), Quil-A was retained by the filter (unpublished data).

A new sensor developed in Professor Anders Kristensen's group at DTU Nanotech was used by Marco Crosio and Kristian Tølbøl Sørensen to measure Quil-A release from the

monoolein-based particles with Quil-A from Paper I (Figure 15, unpublished data). The results showed that Quil-A is released almost immediately after reconstitution. It must be stressed that this measurement was performed without the 20 min re-dispersion step used for the measurement of OVA release in Paper I and for all *in vivo* studies. It thereby indicates that Quil-A has been released completely prior to injection during the *in vivo* studies. It should be noted, however, that the measurement had to be done on particles without OVA. As these are mainly of vesicular structure rather than cubosomes (Paper I), the results must be interpreted with caution.

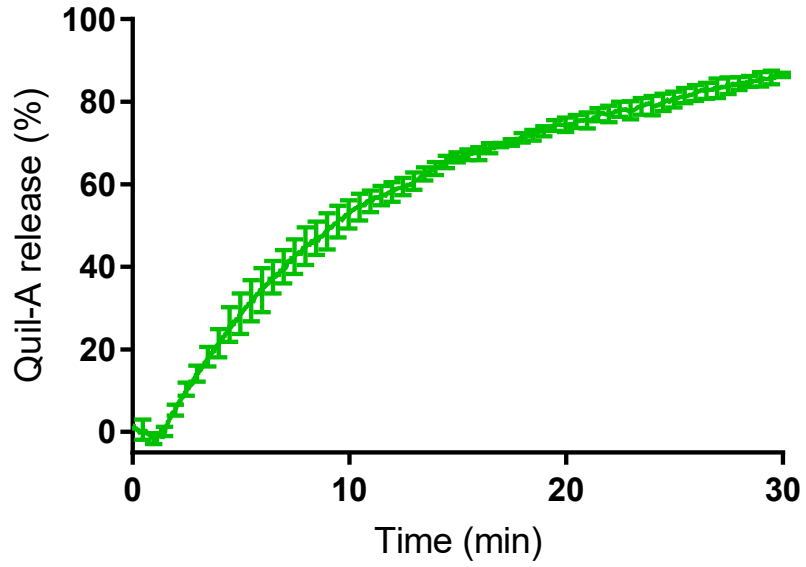


Figure 31. Release of Quil-A from the monoolein-based particles (vesicles) with Quil-A from Paper I. The measurement was performed in Milli-Q water using a photonic crystal slab sensor. Data is represented as mean ± standard deviation (n = 3).

On the assumption that Quil-A is indeed released faster than OVA from cubosomes, it would thus be expected that cubosomes with OVA and Quil-A should be able to stimulate strong humoral and cellular responses (sections 3.3.1.3 and 3.3.2.3). This was indeed observed after s.c. administration in Paper I, but not after oral administration in Paper I or Paper III. The fast release of antigen and adjuvant is a possible reason why the cubosomes have little effect orally, as the great dilution in the GI-tract (section 2.2) might make it

unlikely for individual APCs to be exposed to both OVA, Quil-A and cubosomes if OVA and Quil-A are not physically linked to the cubosomes.

9.3. Microcontainers for oral delivery of cubosomes

Microcontainers were applied to protect the vaccine from chemical and enzymatic degradation in the stomach and reduce the effect of bile salts in the intestine. As discussed in section 2.2, the GI-tract of the mouse varies substantially from that of humans and it also varies between mouse strains. Relying on published human data when designing an oral delivery system for mice is therefore risky. Paper III investigated the pH of the mouse GI-tract to allow rational choice of pH-sensitive polymer to form the microcontainer lids. Eudragit® L100-55 (EL100-55) was chosen as it dissolves at pH above 5.5, which is higher than the gastric pH and lower than the intestinal pH of C57Bl/6 mice (the strain used in these studies). The rate of dissolution of EL100-55 increases with increasing pH and at pH 6, the lids dissolve fast (unpublished observation). This provided release kinetics suitable for the short transit time of microcontainers through the small intestine of the mouse. Dibutyl sebacate (DBS) was added as plasticiser to the EL100-55 to avoid cracking during drying after spray coating of the otherwise brittle lids (Paper III). DBS is hydrophobic and insoluble in water. The use of DBS to plasticise Eudragit® E100 has been reported to result in a material that is more difficult to hydrate than when using a more hydrophilic plasticiser [8]. DBS was therefore chosen to plasticise the microcontainer lids in order to achieve lids with low water permeability.

Despite the promise of microcontainers *in vitro* (discussed in Paper III), *in vivo* results were disappointing. Microcontainers not only did not improve the immune response stimulated by cubosomes with OVA and Quil-A after oral administration, but reduced it to the level of the negative control without OVA. This could indicate that there is an issue with the release of cubosome precursors from the microcontainers or with the generation of dispersed particles following release. Observations in paper III indicate that cubosome precursors should be released rapidly from microcontainers once they reach the small intestine and that the release kinetics of cubosome precursors from microcontainers was adequately fast. Recalling the discussion of section 9.1, it is therefore plausible that formation or dispersion of particles might be an important factor in explaining the disappointing results with microcontainers.

Doherty et al. reported on a vaccine that was unable to prime an immune response when administered orally, but stimulated strong protective immune responses in the lung after s.c. prime followed by an oral booster [9]. The booster was known to be important for the immune response since a single s.c. administration was ineffective [9]. This led us to investigate if microcontainers with cubosomes with OVA and Quil-A could be effective orally as booster vaccines. However, these boosters could not stimulate a cellular response and the humoral responses, both systemic and mucosal, were weak and inconsistent (Paper III). In this study though, the oral dose was the same as the s.c. dose. This might have been too optimistic as oral vaccination generally requires increased doses [10].

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10. General conclusions

The main goal of this thesis was to evaluate microcontainers as a system for oral vaccine delivery. For this purpose, a vaccine in powder form was needed and the creation of a model vaccine in powder form was therefore the first goal of this PhD. Powder precursors of cubosomes with OVA and Quil-A could be produced in a fast and up-scalable manner through a simple spray drying process. The method was robust with the morphology and size of the hydrated particles being independent of the spray drying parameters within a wide range of settings. The spray drying parameters can therefore be adjusted to obtain minimal residual moisture, high powder yield and, if relevant, a low outlet temperature.

The secondary structure of OVA was unaffected by the spray drying process. Furthermore, the powder could be stored for 6 months at room temperature and heated to 86°C for 24 h without affecting the secondary structure of OVA. The powder formed cubosomes in the nano-size range after reconstitution and the cubosome structure was stable over time in aqueous dispersion although the particles showed limited colloidal stability. The adjuvant Quil-A did not affect the structure of the particles despite its surfactant properties and a large content of OVA could be included in the powder. However, some of it may have been freely present in the powder as it was freely present outside the particles after reconstitution and dispersion.

Hydrated cubosomes stimulated strong humoral and cellular immune responses in mice after s.c. administration but not after oral administration. Microcontainers were evaluated as oral delivery system and appeared promising *in vitro* being able to encapsulate the cubosomes at stomach pH and rapidly release them at intestinal pH. The fast release was important due to a short intestinal residence time of the microcontainers in mice. Nevertheless, *in vivo* results were disappointing and indicated together with the *in vitro* characteristics of the cubosomes that a better vaccine needs to be used for delivery in the microcontainers. An important characteristic to improve include the ability of the vaccine powder to reconstitute and disperse spontaneously upon addition of water into colloidally stable nanoparticles. Other factors that may be important are prevention of separation of antigen and adjuvant from the particle and improved stability of the particle in the intestine after release from the microcontainers.

11. Future perspectives

One of the main drawbacks of the spray dried cubosome vaccine was its poor ability to reconstitute and disperse into small particles. Adding a better colloidal stabiliser such as Pluronic 127 is an important future direction for spray dried cubosomes. Ingvarsson et al. found that trehalose is a good stabiliser for preformed liposomes during spray drying [1]. Trehalose is also reported to be a good stabiliser for proteins [2] and thus changing dextran with trehalose might be beneficial in later studies, especially for use with more fragile proteins/peptides than OVA.

Quil-A is not suitable for human vaccines due to limited natural resources, batch-to-batch differences and toxicity. Replacing it with a safer and more reproducible alternative such as a synthetic analogue of QS-21 would be preferable. For improved immunogenicity, it should be considered to join QS-21 with other immunopotentiators such as MPLA. Cubosomes also may not be the best particle for oral delivery with microcontainers due to their sensitivity to bile salts and their fast release of antigen and adjuvant. Evaluation of the importance of physical linkage of antigen and adjuvant to the particles in an oral setting would be an important contribution to the future design of oral vaccines. An interesting alternative to cubosomes is VLPs formulated to prevent rapid release of antigen and adjuvant. VLPs may be composed of capsid proteins of an enteric virus and thus might be more stable than cubosomes in the small intestine. In addition, as VLPs are preformed particles, their behaviour might be more predictable *in vivo* than cubosomes.

The microcontainers used in these studies were microfabricated in a cleanroom from the negative epoxy photoresist SU-8 using a photolithography process. This method is not compatible with the inexpensive large scale manufacturing generally required for commercial vaccines and thus must be replaced. Roll-to-roll manufacturing is currently being pursued in our group to address this issue. The microcontainers used in this study are furthermore not biodegradable. This did not seem to cause any adverse effects in the mice during the studies of this PhD as the microcontainers were simply defecated along with other indigestible particles that the mice had ingested. However, it may complicate licensing by regulatory authorities. It may therefore be preferable to make microcontainers from a biodegradable polymer that has already been used in licensed products such as PLGA.

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